ALGENOL

Algenol Integrated Pilot-Scale Biorefinery

January 29, 2010 – July 1, 2015

Public Version Final Report

Project Title:	Recovery Act – Integrated Pilot-Scale Biorefinery for Producing Ethanol from Hybrid Algae
Award number:	DE – EE0002867
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Project Partners:	National Renewable Energy Laboratory (NREL), Georgia Institute of Technology (GaTech), Membrane Technology and Research (MTR), The Dow Chemical Company
Report Date:	May 26, 2017
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Table of Contents

Forward

Message from Algenol

Algenol's investors, management and employees would like to thank the U.S. Congress, the President of the United States and the U.S. Department of Energy for providing Algenol the opportunity to work under the Demonstration of Integrated Biorefinery Operations grant program. We would especially like to thank Jonathan Male, Jim Spaeth, Alison Goss-Eng, Christy Sterner and the rest of the professional staff at the DOE for their support of Algenol over the past seven years. Your understanding of the challenges we encountered during this program, and your unwavering support through it all, was welcomed encouragement to push forward, solve problems and lay the technological groundwork on which to build a new industry.

Programs such as this one are very important for the development of nascent technologies like ours. Being awarded the DOE grant and building the biorefinery greatly assisted Algenol in attracting the investment capital necessary to advance its technology. Our investors have invested over 14x the amount of the DOE grant, which is a testament to this fact.

This program had audacious goals to be sure; take waste carbon dioxide from anthropogenic sources, feed it to microalgae being cultivated on a massive scale, reduce by at least 60% the carbon footprint of the process over gasoline and try to compete with a fuels industry that has a 100-year head start and enormous human, technical and financial resources. Given the current global prices, it is not currently feasible to be cost competitive with crude oil or attract the necessary capital to continue a large biofuels research and development program. Nonetheless, the fundamental ground work for the technology has been demonstrated, and we know that producing algal biofuels is well within the realm of reality. We believe we are within striking distance of economic viability for the Direct to Ethanol® process. As with any significant technological development, however, achieving this goal will take more time, intellectual resources and money, but this grant has helped to position us well to succeed when energy market conditions become more favorable to biofuels.

Whereas we have not yet achieved economic viability to compete with cheap crude oil, we did develop a disruptive technology for application in other markets beyond the biofuels sector. Two key areas of high tech innovation are 1) our ability to bioengineer a variety of sustainable bioproducts from various algae and cyanobacteria and 2) our development of an advanced photobioreactor system competitive with and more productive than traditional open pond cultivation systems. We will move into large sustainability driven, natural ingredient markets in which unique algae attributes and the benefits of our cultivation technology give Algenol a distinct competitive advantage. The innovative technologies, infrastructure and knowhow we developed now allow us to compete in many attractive markets supplied by firms that rely on cheap overseas labor as a competitive advantage. We intend to disrupt these markets with our products, and by doing so continue to provide high paying domestic jobs in product R&D and to create additional skilled jobs in U.S. rural agricultural communities as our bioproduct facilities come online.

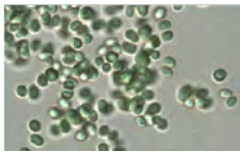
We are going to continue our efforts to develop a sustainable algal based biofuel, albeit at a more limited pace, and we will continue to scale up our technology in other markets. We will be ready to deploy our biofuels technology when market conditions improve and when decision makers realize that waste carbon dioxide from industrial sources has significant value and should be recycled into valuable products and not dumped into the atmosphere where it creates no value for anyone.

The Algenol Team

Algenol Company Overview

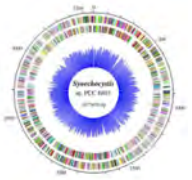
Algenol Biotech, LLC, (Algenol) is an industrial biotech company offering new approaches for biobased products utilizing algae. Algenol operates in algae-based agtech, biofuels and contract research, development and manufacturing sectors. Algenol was formed in 2006 and is headquartered in Fort Myers, Florida and has an R&D facility in Berlin, Germany.

The key strength of Algenol is its employees. Algenol has approximately 120 employees with 100 in Fort Myers and 20 in Berlin. Algenol's professional staff has extensive experience in all aspects of algal research, strain improvement, cultivation and process development. Over 70% of Algenol staff have Bachelors or Graduate degrees and extensive experience in biology, algal sciences, chemistry, engineering, finance, business and law.



Algenol has extensive algal research and development capabilities. Algenol has over 2,000 algal strains in its culture collection to meet most any commercial purpose. This strain collection has unicellular and filamentous algal strains from diverse environments, and we have access to thousands of additional strains through multiple culture collections.

Algenol has unmatched experience in all aspects of algal molecular biology for developing cyanobacteria as heterologous expression systems for a broad range of products such as ethanol, proteins, enzymes, nutritional supplements and biochemicals. Algenol's scientists have developed a complete molecular biology tool box for multiple strains including plasmid vector construction, transformation, homologous recombination and gene expression control. Algenol scientists utilize targeted and non-targeted approaches for strain development, and can stack multiple desirable traits in a single production cell line. In addition, screens, selections, and adaptation are used to identify strains with improved properties. Modifications to known genes can be made to target a specific improvement in a strain. Modern 'omics technologies are powerful methods for gene discovery and strain characterization and are employed by our scientists to inform strain development strategies and to create desired production cell lines.



Algenol's Fort Myers campus has extensive facilities to conduct all aspects of algal research, development and cultivation. Algenol's facilities consists of 30,000 sq. ft. state-of-the-art research and development laboratories dedicated to algae. There are four separate scientific labs, each dedicated to different areas of algal research. Algenol has extensive lab-based algae screening capabilities, strain adaptation capabilities and complete lab-based process development capabilities. The lab facility also has six dedicated algae cultivation rooms with computer-based temperature control to simulate a daily range from 8 °C to 45 °C. These cultivation rooms have sophisticated lab photobioreactors (PBRs) scaled to mimic commercial PBRs for relevant lab to outdoor growth comparisons, allowing rapid process development. This facility also has a fully equipped photosynthesis laboratory to conduct rapid testing of cell line performance under any light intensity and accurately measure productivity at the cellular level. This facility has a carbon-14 license and carbon-13 capabilities.

This facility has a state-of-the-art analytical chemistry lab to measure all relevant process parameters and cellular constituents. This lab has a professional analytical chemistry staff that delivers test results for quality control to meet the highest standards. All data generated by the

lab is managed by a Laboratory Information Management System (LIMS). Algenol has made a significant investment in automation and auto sampling.

- Analytical Chemistry Lab Equipment List
  - LC-MS/MS (Q-ToF)
  - GC (4), HPLC, GC-MS, IC and KF
  - UV/Vis Spectrophotometer, Organic Elemental Analyzer, Organic Carbon Analyzer, Thermal Analyzer, Spectrofluorometer, ICP Spectrometer, Nutrient Analyzer

Algenol's outdoor research capabilities are unmatched anywhere. Algenol's Process Development Unit (PDU) is a 4.5-acre greenfield facility designed specifically for algae cultivation, R&D and commercial scale up. This facility is licensed by the Florida Department of Aquaculture for use in experimental research utilizing both native and genetically modified algae. This facility is designed for large-scale outdoor process scale-up using commercial scale PBRs for relevant process development. This facility has the capacity to test multiple algae strains and PBR experiments run in parallel. The facility has flexible cultivation systems that allow on-the-fly process changes to optimize culture conditions. Algenol developed a proprietary data acquisition and control system that collects relevant ambient weather and light data, monitors all PBR process data, provides automated alarms that alert production personnel of process upsets and uploads all data to a database for further analyzation and trending.

Funding from DOE supported the construction of the Integrated Biorefinery (IBR) at the Fort Myers campus. As a true integrated pilot plant, the IBR greatly expands Algenol's capabilities to commercialize algae-based products at all R&D scales, from petri plate to plant, all at one site. The DOE grant that supported the construction and operation of the IBR is the topic of this report.

Algenol has 44 patents and approximately 38 pending patent applications for key technologies in its portfolio. Key technology areas cover by this patent estate are:

- Commercial algae adapted to make high-value products
- Photobioreactor cultivation systems for algae
- Low-cost product capture and purification systems
- Enzyme systems in algae for production of biofuels
- Next-generation photobioreactor systems
- Metabolically-enhanced productive algae
- Plant-scale production systems utilizing carbon dioxide feedstock
- Stability enhancements in productive algae

Direct-to-Ethanol® and VIPER Photobioreactor Technologies

Direct-to-Ethanol® – Algenol's Direct to Ethanol® technology is a unique integrated process for production of liquid biofuels. This process utilizes a novel and proprietary algal strain that is referred to as AB1. Algenol's technology uses carbon dioxide (CO_2), salt water, sunshine and electricity to enable the growth of AB1. The Direct to Ethanol® technology involves over-expressing in blue-green algae (cyanobacteria) the genes for fermentation pathway enzymes found widely in nature. These enzymes are pyruvate decarboxylase (PDC) and alcohol

dehydrogenase (ADH*)*. The resulting metabolically enhanced hybrid algae actively carry out photosynthesis and utilize carbon dioxide as the feedstock for producing and secreting ethanol from each algal cell.

The key to the productivity of Algenol's technology is the production of ethanol and biomass at the same time using the same organism and the subsequent conversion of the biomass into bio-crude oil, thus allowing the conversion of over 85% of the introduced carbon into transportation fuel. Algenol's technology consumes and recycles CO_2, a greenhouse gas that would otherwise be emitted into the atmosphere, as an input feedstock, and converts algae into valuable transportation fuel in an energy-efficient manner. For every gallon of Algenol's ethanol being used, approximately 2/3 gallon of fossil-fuel gasoline is displaced.

Algenol's Direct to Ethanol® technology has three core components:

1. World's Most Productive Algae Platform
   - Proprietary enhanced algae make ethanol and biomass directly from CO_2, water, and sunlight.
   - Spent algae are converted into biodiesel, gasoline, and jet fuel.
   - 85% of the CO_2 is converted into products.
2. Specialized VIPER™ PBRs
   - Algae are grown in salt water contained in proprietary PBRs that are exposed to the sun and are fed CO_2 and nutrients.
   - A production cycle runs for 3 to 6 weeks or longer
   - Afterwards, the spent algae are separated from the water-ethanol mixture.
3. Energy Efficient Downstream Processing
   - Water-ethanol mixture is sent to proprietary downstream processing equipment that separates and concentrates it to fuel grade ethanol.

Algenol's technological process will give large fossil fuel-based CO_2 emitters the ability to monetize their waste carbon dioxide streams and therefore turn an environmental liability into a valuable asset. The beneficial reuse of fossil fuel-based CO_2 through this novel technological process has the ability to dramatically alter the economics around CO_2 reduction for large fossil fuel-based carbon emitters in various industries.

In addition to the benefits of CO_2 emissions reduction, the Direct-to-Ethanol® technology does not require use of high-value land that can be used for productive purposes such as farming. Because the production process uses innovative clear plastic PBRs that are hung from support systems, low-cost, non-productive land can be utilized for growing the algae. The land could be situated next to an industrial CO_2 source, so CO_2 that would otherwise be vented or sequestered at a high cost would be recycled into, ultimately, the production of four important transportation fuels.

VIPER Photobioreactors – Algenol's VIPER™ photobioreactors are low cost systems that open up new opportunities for profitable large-scale algae cultivation. Each PBR maximizes light distribution and moderates temperature for maximum algal production yields. Viper PBRs efficiently deliver carbon dioxide and nutrients to the algal culture allowing tight control over the culture conditions permitting better control over desired algae composition and providing maximal productivity. The VIPER system allows highly efficient algae growth at 2–3x productivity over open pond systems. VIPER PBRs limit contamination from other algae species to maintain monocultures for the highest quality algal product and fewer system upsets from invading organisms. VIPER systems have greater product concentrations at harvest compared to open ponds. These systems can have a high degree of automation to control operational costs. VIPER systems have proven effectiveness across broad range of algae types.

Algenol has built a large-scale state-of-the-art VIPER plastics manufacturing and assembly facility for commercial manufacture of PBRs. Algenol uses Finite Element Analysis and Computational Fluid Dynamics software to design high performance PBRs that are durable and have long in-the-field lifetime. Algenol works with multiple large plastics companies to develop plastic formulations for high performance PBR films. Algenol utilizes cutting edge plastics testing and accelerated aging technology to evaluate long term endurance of the VIPER systems.

Project Partners

National Renewable Energy Laboratory (NREL), Georgia Institute of Technology (GaTech), Membrane Technology and Research (MTR), The Dow Chemical Company

Abbreviations and Acronyms

ABCC – Algenol Biotech Culture Collection

ADH – alcohol dehydrogenase

ADP – adenosine diphosphate

ATP – adenosine triphosphate

APHIS – Animal and Plant Health Inspection Service

ASME – American Society of Mechanical Engineers

ASTM – American Society for Testing and Materials

BTU – British thermal unit

CBB – Calvin Benson Bassham cycle

CET – cyclic electron transport

CFD – computational fluid dynamics

CIP – clean-in-place

CMO – contract manufacturing organization

CO – carbon monoxide

CO_2 – carbon dioxide

cRIO – compact reconfigurable input output module

cu ft – cubic feet

DNA – deoxyribonucleic acid

DOC – dissolved organic carbon

DOE – Department of Energy

DW – dry weight

EA – Environmental Assessment

EIR – Environmental Impact Report

EIS – Environmental Impact Statement

EH&S - Environmental, Health and Safety

EPA – Environmental Protection Agency

EPS - exopolysacchides

EtOH – ethanol

GAP - glyceraldehyde-3-P

GAPDH – glyceraldehyde-3-P dehydrogenase

GC – gas chromatograph

GC-FID – gas chromatography with flame ionization detector

gal – gallon

GEPAY – gallons of ethanol per acre per year

GHG - greenhouse gas

GMO – genetically modified organism

GPM – gallons per minute

HAZOP – hazard and operability study

HDPE – high density polyethylene

HMI – human-machine interface

IBR – integrated biorefinery

IGT – Internal Gatekeeper Team

IPC – in-process control

kg – kilograms

kW – kilowatts

kWh – kilowatt-hour

lbs – pounds

LCA – life cycle analysis

LET – linear electron transport

LLDPE – linear low density polyethylene

MCAN – Microbial Commercial Activity Notice

MDH – malate dehydrogenase

ME – malic enzyme

MSDS – Material Safety Data Sheet

mt – metric tons

NADPH – nicotinamide adenine dinucleotide phosphate

NEPA – National Environmental Policy Act

NO_x – nitrogen oxides

NREL – National Renewable Energy Laboratory

OAA – oxaloacetate

OEM – original equipment manufacturer

OPP – oxidative pentose phosphate

ORF – open reading frame

OSHA – Occupational Safety and Health Administration

PAM – Project Accounting Manager

PAR – photosynthetically active radiation

PBR – photobioreactor

PC – phycocyanin

PDC – pyruvate decarboxylase

PDH – pyruvate dehydrogenase

PDU – process development unit

PE – polyethylene

PEP – phosphoenolpyruvate

PEPC – phosphoenolpyruvate carboxylase

PEPP – phosphoenolpyruvate phosphatase

PET – photosynthetic electron transport

3-PGA – 3-phosphglycerate

PGK – phosphoglycerate kinase

PGM – Phosphoglycerate mutase

PK – pyruvate kinase

PLC – programmable logic controller

PMB – Performance Management Baseline

PMP – project management plan

PMT – Program Management Team

PPE – personal protective equipment

PQ – plastoquinone

PRK – phosphoribulose kinase

PSI – photosystem I

PSII – photosystem II

psig – pounds per square inch gauge

PVC – polyvinyl chloride

QC – quality control

R&D – research and development

RBS – ribosome binding site

RCA – Root Cause Analysis

RET – respiratory electron transport

RFS – Renewable Fuel Standard

RIN – Renewable Identification Number

RNA – ribonucleic acid

RO – reverse osmosis

RODI – reverse osmosis/de-ionized

ROS – reactive oxygen species

Rubisco – ribulose-bisphosphate carboxylase/oxygenase

RSD – relative standard deviation

SAT – site acceptance testing *or* Strain Advancement Team

SCADA – supervisory control and data acquisition

SCFM – standard cubic feet per minute

SLPM - standard liters per minute

sOD – standard optical density

SOP – standard operating procedure

TCA – tricarboxylic acid

TGOLF – total gallons of liquid fuel (per acre per year)

TOC – total organic carbon

TPP – thiamine pyrophosphate

TSCA – Toxic Substances Control Act

TTB – (Alcohol and Tobacco) Tax and Trade Bureau

UV – ultraviolet radiation

USDA - U.S. Department of Agriculture

VCSS – vapor compression steam stripper

VLE – vapor liquid equilibrium

VOCs – volatile organic compounds

v/v – volume/volume

WBS – work breakdown structure

w/w – weight/weight

List of Figures

List of Tables

Executive Summary

As a scientific and engineering endeavor, the Algenol IBR Biorefinery project has been a success by almost any measure. The vision for the system evolved significantly over the course of the project, always due to recognized opportunities for improved performance, lower energy consumption, and reduced costs. Our commitment to thorough, realistic, techno-economic and life cycle assessments has been an essential element for system innovation, technology guidance, and change management of the overall facility. The biological tools developed during this program for cyanobacteria are second to none, and are the primary reason for the remarkable improvements in organism performance. The breakthrough was the successful transformation of our most robust wild type organism (AB1) early in 2012. That was followed by a series of improvements over the next several years that produced strains wherein over 80% of the fixed carbon was converted into ethanol. At the same time, our expertise in cultivation, physiology, process engineering, CO_2 management, and photobioreactor design and manufacturing grew at a comparable pace. We learned enormous amounts from the various upsets, weather variations, contamination events, and new technology related disappointments. We overcame those challenges to produce fuel grade ethanol with a low carbon footprint, and are within striking distance of economic viability even with the challenges of low fossil fuel prices.

Advances in Biology – Algenol's extensive experience in cyanobacterial strain development has led to better and better strains, not only from the perspective of increasing ethanol productivity, but also with respect to traits that will enable commercial production, such as enhanced genetic stability and contamination control. Significant productivity increases have largely come about via improvements in the ethanol cassette, which have led to the diversion of a very high proportion of the fixed carbon into ethanol such that over the past four years, ethanol productivity rates for AB1 derivatives under standard laboratory conditions have increased five-fold. Algenol molecular biologists are now focused on further enhancing the production strain to improve overall photosynthetic carbon fixation rates, where we believe significant additional increases in productivity can be realized.

Genetic stability was identified as a major factor for duration of ethanol productivity for outdoor cultivation. Culture stress from introduction of the ethanol cassette and the resulting production of acetaldehyde and ethanol combined with the higher growth rates for non-ethanologenic cells can lead to favored proliferation of mutated "revertant" cells that have lost their ability to produce ethanol. Colony size analyses and small-scale ethanol production assays were developed which allowed for an understanding of population dynamics of ethanol-producing and non-producing revertant cells. These studies led to improvements in repression of the expression of the ethanol gene cassette during inoculum production, as well as recommended guidelines for culture quality parameters, such as the allowable percentage of revertants in an inoculum and the recommended number of generations for semi-continuous operations.

Advances in Operations – Algenol's cumulative cultivation experience has led to the development of a comprehensive set of recommended cultivation standards for maximal ethanol yield. This set of standards not only lays out basic strain requirements through culture medium optimization, but also simplifies operations through bulk additions of nutrients based on seasonal growth models. Improvements to inoculum quality by reducing inoculum harvest densities as recommended in the cultivation standards led to more robust cultures and decreased inoculation growth lags.

Ozone was initially found to be a very effective sterilization method for both PBR systems and well saline water. However, additional clean-in-place (CIP) strategies were needed as

research demonstrated long-term material damage from ozone as well as the need for liquid chemical cleaning methods to remove inorganic deposits and residual biomass between batches. The maximum acceptable contaminant level (MACL) criterion was developed similar to EPA drinking water standards as a way of evaluating CIP effectiveness. Through an extensive and iterative series of experiments on chemical selection, cleaning order, and cost-effectiveness, a final "robust" CIP method was developed that employed a caustic/bleach step to disrupt biofilms and an acid step to remove inorganic crusts and disinfect the system.

An additional cost-cutting exercise explored the feasibility of developing mutually beneficial partnerships with utility companies for industrial gas supply. Flue gas from both natural gas and coal-fired power plants is a potentially inexpensive source of CO_2 for commercial production of algal-derived biofuels. Algenol worked with a Florida power producer to evaluate production of ethanol and biomass using flue gas as the primary source of CO_2 supplied to the algae at lab scale. These experiments were conducted under standard operating conditions designed to replicate annual average outdoor conditions in Fort Myers, Florida with regard to temperature and light. Productivities for flue gas treatments were compared to controls using diluted pure CO_2 as a carbon source. The observed biomass and ethanol productivity were comparable to those for outdoor cultivations for both the flue gas treatments and the pure CO_2 controls.

Advances in Engineering – A pivotal moment for Algenol came with the switch from horizontal to vertical photobioreactor platforms. Vertically-oriented systems were proven to solve four main challenges identified from horizontal designs, namely inefficient light utilization, high culture temperatures, high oxygen concentrations, and culture genetic instability, which allowed for large gains in productivity. Furthermore, vertical PBRs afforded economic gains from their ability to be inoculated at lower cell densities and use lower water volumes for a given production area and improved downstream processing efficiency by achieving higher harvest ethanol concentrations compared to horizontal systems. Finally, the integration of PBRs and associated piping into large-volume airlift arrays led to improvements in nutrient delivery, circulation, cleanability, and ease of operation while also cutting costs through minimization of ports and other fittings.

In 2014 Algenol conducted a thorough study to determine the true cost of goods for its vertical photobioreactor platform. These costs were determined based on high volume production and deployment of the existing PBR system (VIPER 2.3). All components of the system were well defined in the study and included all film, welds, tubing, support structure, and other associated components dictated by the current PBR design. In its original state at the 1.8-acre IBR, the cultivation system was estimated at $132.68 per 10-ft PBR. The study revealed that this cost could be reduced to $100.70 based on volume and vendor-based discounts according to actual quotes. Most of the reduction resulted from film optimization, which decreased film costs from $29.22 to $12.97 per 10-ft PBR. Little gain was derived from volume discounts on the remainder of the system, i.e. support structure and piping. Based on these findings, a program was developed to achieve further cost improvement through design innovation and optimization. The program focused on the following:

- Reducing material waste and making better use of pre-fabricated components for the support structure.
- Working with vendors to develop the required material performance across the system.
- Adopting more efficient manufacturing techniques, for example, laser drilling on the extrusion line instead of manual punching for air diffuser tube production.
- Developing thinner piping with better barb attachment, including existing integrated welded outlets.

- Minimizing the need for the support structure by considering self-supporting configurations.

In addition, the team worked with plastic film vendors to develop films with longer deployable lifetimes under outdoor conditions. In the program, Algenol enlisted the services of multiple resources, including polymer suppliers, additive suppliers and blown film manufacturing companies to develop a long life, tough, weldable, clear, biocompatible, low creep, and flexible film for PBR construction. The best candidates of these films have been subjected to the equivalent of 6 years of UV exposure in UV chambers and maintain sufficient performance to provide high confidence in their outdoor performance capability. Higher strength UV stable materials with lower gauge thicknesses have been identified which will serve to improve the economics of future PBR designs.

Based on these approaches, the program delivered 83% and 61% projected cost reduction in support structure and piping cost respectively, bringing the cultivation system cost from its original $100.7 down to $30.96. Further optimization in design would result in a system cost of $26.64 per 10-ft PBR, representing an overall total 74% cost of goods reduction from 2014 to date. Future cost projections in film and elimination of the support structure could lead to significant additional cost reduction.

Algenol built and developed an in-house manufacturing subsidiary, Viper Co., to manufacture prototype and commercial PBRs, and this capability led to substantial improvements in PBR reliability and quality—an achievement that had cascading benefits for operational stability and contamination control.

Algenol's process engineering has developed and piloted a downstream ethanol extraction process that meets basic commercial design requirements and energy expenditures for a viable commercial product. The key innovation is the proprietary VCSS (Vapor Compression Steam Stripper), a highly heat-integrated technology that provides for energy efficient ethanol extraction from a salt water medium with low ethanol concentrations. Conventional ethanol extraction technologies at this first extraction step require more energy input than the energy recoverable in ethanol. Nonetheless, the VCSS provides a 10-fold increase in concentration, which when combined with more traditional extraction equipment provides an integrated ethanol purification system. Algenol has piloted the integrated downstream ethanol purification system at the IBR, and has demonstrated fuel-grade ethanol, as certified by an independent laboratory, derived from the cyanobacteria.

Working with State and Federal Regulators – First and foremost, Algenol prides itself on developing safe, sustainable and environmentally friendly products, including biofuels, and has worked closely with state and federal agencies to ensure that all regulatory requirements are met. One requirement for the IBR project was that Algenol needed an aquaculture license from the Florida Department of Agriculture and Consumer Services (FDACS), but additional strain specific approvals were required to deploy the Company's enhanced hybrid algae. To date, several strains have been approved for deployment at the IBR, including derivatives of AB1, the strains identified for potential commercial deployment. These approvals were based on an initial screening paradigm designed to eliminate potentially harmful or invasive strains as well as a series of robust environmental studies that were designed, and in some cases conducted, with significant oversight from FDACS. Of particular importance were: 1) evidence that Algenol's production strains do not produce toxins (as assessed by whole genome sequence analysis and liquid chromatography-mass spectroscopic analyses), 2) demonstration that the strains were non-invasive in a variety of local water types with varied chemical composition and salinity, and 3) determination that the strains were not plant pests. Algenol took the additional step of obtaining an exemption for AB1 from certain special state permitting requirements for non-native

species, which was done through a formal rulemaking process and is now codified in Florida statute.

As the Company transitioned to pilot-scale production and commercial demonstration activities, it was necessary to file a Microbial Commercial Activity Notice (MCAN) with the US EPA as required by the Toxic Substances Control Act. The process for completing the MCAN and negotiating the corresponding Consent Decree was a comprehensive process that took more than a year, starting with pre-submission notification meetings between Algenol and EPA to identify submission requirements. The MCAN that Algenol submitted described every aspect of the overall manufacturing process in addition to highly detailed information regarding the host algal strain and the genetic enhancements executed by Algenol's scientists. Also included were the many environmental studies Algenol had conducted in order to obtain approvals from the state of Florida. In December 2014, Algenol and EPA agreed on a Consent Decree that allowed the Company to initiate commercial operations at its Fort Myers, FL facility as requested in the submitted MCAN. Also negotiated into the Consent Decree was a roadmap for future MCAN approvals including reasonable information EPA requested in order to approve an MCAN for a commercial scale project.

In July of 2014 Algenol petitioned the EPA for approval of a pathway for the generation of advanced biofuel Renewable Identification Numbers (RINs) consistent with the requirements of the Renewable Fuel Standard program. The new pathway would cover ethanol produced by photosynthetic cyanobacteria using Algenol's proprietary Direct to Ethanol® process. In the petition Algenol also described its ability and intent to produce a bio-oil co-product and took the position that an appropriate pathway already exists that will allow the Company to generate corresponding RINs. On December 2, 2014 EPA issued an approval of the requested new pathway and agreed, in writing, with Algenol's assertion that an appropriate pathway exists for the biocrude oil it will produce along with ethanol. As required by statute, the EPA conducted a life cycle analysis of Algenol's process and stated in their approval documentation that ethanol produced through the Algenol pathway reduces lifecycle greenhouse gas emissions compared to the statutory petroleum baseline by 69%. This allows for the generation of advanced biofuel, or D-Code 5, RINs.

Stability and Ethanol Production at the IBR – The aforementioned technological and economical breakthroughs culminated in the design, deployment, and operation of the Integrated Biorefinery. Over several months in the latter portion of 2014, a process pavilion, process pad, scale-up pad, and supports for the PBR field were constructed. PBRs were set up in integrated blocks, with a final deployment of 8 blocks of 408 integrated VIPER 2.3 PBRs at a base spacing, 5 blocks of 408 integrated VIPER 2.3 at a different spacing, and 2 blocks of 200 VIPER 3.1 airlift systems at the base spacing. Cultivation began in early 2015, with all VIPER 2.3 blocks operational by May 2015. Initial batches suffered from culture stress and grazing; however, an exhaustive current state analysis was performed and risks to stability were identified. The products of these activities were three main recommendations: 1) resolve impact of ciliates on batch productivity by eliminating PBR field leaks, implementing routine salt water tank CIPs, fixing filtration system problems, and implementing secondary ciliate control with quinine sulfate; 2) minimize negative impacts of early culture stress and late stage ethanol consumption by lowering inoculum harvest density, improving CO_2 control through advanced control systems, control P-delivery by implementing seasonal P-dosing schedules, and improve growth lag times by introducing CIP chemical residue monitoring with added rinse steps; and 3) reduce mechanical and human errors by finalizing operational checklists and maintenance records and implementing management of change procedures. By following these recommendations, we were able to run 9 total consecutive batches for a final demonstration of stable operations with consistent ethanol production.

As program activities came to a close, productivities across scales for batches without special cause variations (ethanol consumption, growth lags, etc.) typically reached or exceeded 5,000 TGOLF (Figure ES-1), similar to indoor cultivations and demonstrating the capability of the technology to reach scale. Minimization of inoculation stress and advanced contamination control strategies for eliminating ethanol consumption will be the primary focus areas for future cultivation research to ensure batch to batch consistency and maintenance of high productivity rates.

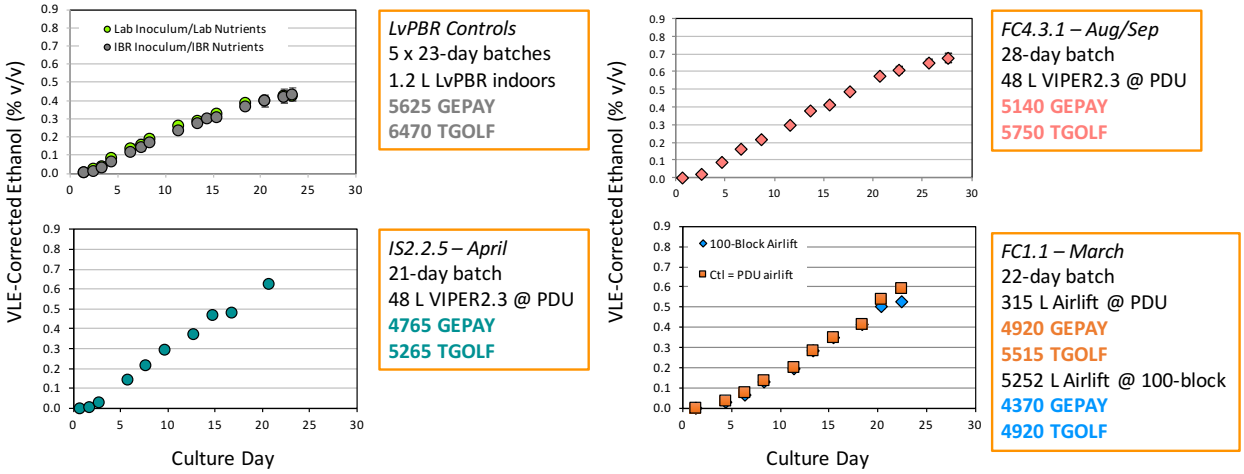


Figure ES-1. VLE-corrected ethanol and annualized productivity rates for experiments across varying scales, including results from the Integrated Biorefinery. The selected experiments were not impacted by special cause variations such as ethanol consumption or inoculation lags, and represent base case productivities for routine batches across scales.

Process, Economic and Life Cycle Modeling – Since the first cultivation studies in 2008, fundamental understanding of cyanobacteria ethanol production has been developed through both experimental research and process modeling. Algenol has developed a numerical culture growth and ethanol production model that can simulate process dynamics from small scale bottle reactors in the laboratory, to intermediate prototype PBR platforms under outdoor conditions, and finally to large commercial systems. The model is useful for investigating the complex interactions between biological (i.e., photosynthesis and ethanol metabolism), chemical (i.e., CO_2 uptake) and physical (i.e., sunlight and temperature) processes that occur in PBRs under real-world conditions. The Production Model is used in three fundamental ways: 1) model simulations provide insight into culture growth and ethanol production and result in basic research guidance and optimization recommendations, 2) the model has been integrated into CO_2 delivery algorithms and other controls systems processes, and 3) the model is used to extrapolate experiment results to annual production expectations for a commercial plant, potentially located anywhere in the world practical for an algae facility.

In parallel, a strong commitment to Techno-Economic Analysis (TEA) has been an integral part of Algenol's technical portfolio. As a novel technology, the earliest versions of the TEA for the Direct-to-Ethanol® process were rudimentary because so many of the engineering and biological systems were first-of-kind. However, the TEA results were compelling enough to attract private equity funding sufficient to enable proof of concept and provide the matching funds needed for this DOE project. The underlying physical system that formed the basis for the TEA also formed the basis for the Life Cycle Analysis (LCA) which showed that Direct to Ethanol® could provide a very significant reduction in CO_2 footprint compared to gasoline, well beyond the requirements for this DOE project.

TEA analysis, combined with Algenol's financial model, provides an economic assessment of the viability of the Direct to Ethanol® technology under various scenarios for future economic conditions, primarily oil prices, but also including government incentives for carbon footprint reduction for transportation fuels. In that same context, the TEA with its Capital Expense (CAPEX) and Operating Expense (OPEX) goals provides research guidance for both performance improvements and cost reductions. The TEA has been used in that manner from Algenol's beginnings and throughout the execution of the IBR project. From the tornado graph analysis, the major economic impacts originate from productivity, PBR cost, PBR lifetime, and CO_2 cost.

Overall the system is close to economic viability based on the final P50 estimates produced by our TEA analysis. Some combination of improved productivity, continued reduction in PBR costs, higher oil prices, and a political climate that essentially guaranteed renewable fuel incentives is needed to attract the investments required for deployment of this technology. Algenol continues to work on technical improvements, as well as diversification into higher value products.

Major Accomplishments

1. Transformation of AB1 to produce ethanologenic strains with high genetic stability and high ethanol branching ratios.
2. Development of photobioreactors that optimize light utilization, yield sustainable performance, and are cost competitive with competing technologies for producing algae.
3. Development of Vapor Compression Steam Stripping (VCSS), an Algenol patented process for energy efficient purification of low concentration ethanol-water mixtures.
4. Peer-reviewed publication of the first life cycle analysis for algal biofuels (70% reduction in carbon footprint compared to gasoline, confirmed by EPA).
5. 2015 Presidential Green Chemistry Prize (sponsored by the American Chemical Society and EPA).
6. Development of an extensive algal science base showing that translation of lab productivity data to large scale outdoor facilities can be essentially quantitative with solid scientific principles and methodologies applied to all aspects of the process.
7. Significant innovation in the integration of CO_2 sources with biorefineries including optimization of co-location opportunities with anthropogenic sources and designs for stand-alone systems that avoid the co-location siting constraint.
8. State-of-the-art Integrated TEA and LCA analysis system allowing assessment of project status and providing research guidance for all aspects of the work program.
9. Establishment of the limiting factors in ethanol and biomass production, and quantification of the improvements required for economic viability.

Task A – Development, Planning, & Preparation of Integrated Biorefinery

Task Objective

During Task A of this project, Algenol's principal activities were related to site preparation, construction planning, development of standard operating procedures and EH&S procedures, refining the risk management plan, recruiting and hiring staff, and obtaining permitting and regulatory approval for construction. Algenol continued R&D activities during this period in order to improve the productivity of the hybrid algae, improve the efficiency and lower the cost of the photobioreactor (PBR), refine the process for operating the integrated biorefinery (IBR), and to initiate the R&D activities at NREL and the Georgia Institute of Technology.

Project Activities

Subtask A.1 Appoint project team

Completed January 2010

The original Program Management Team (PMT) was appointed in February 2010. The PMT was chaired by Dr. Craig Smith, the project Principal Investigator (2010-2012), and co-chaired by Ed Legere (Principal Investigator 2012-2015) and was composed of senior Algenol and Dow Chemical scientific and engineering management. The specific roles and responsibilities of the committee included the following: 1) annual operating and capital equipment budget approval, 2) specification of the delegation of financial authority for program personnel, 3) review and approval of Quarterly and Annual Program Operating Plan, including the objectives for each project team, 4) tracking actual expenditures against budgeted and forecasted expenditures, 5) tracking accomplishments against program plans, 6) review and approval of any proposed changes to the budget forecast or changes to the project team objectives (i.e., change control), 7) designing and implementing the Risk Management Plan, and 8) review and approval of any program reports or public statements about the Program.

At the same time, an Internal Gatekeeper Team (IGT) was formed. The IGT was chaired by Paul Woods, the CEO of Algenol and was composed of two Algenol senior executives and two Dow Chemical senior executives. The IGT was responsible for reviewing the project's status at the conclusion of Phase I and Phase II, respectively. At the end of Phase I and Phase II, the IGT reviewed the project reports prepared by the PMT. After review, the IGT submitted, with appropriate documentation, a recommendation to the DOE regarding the pass or fail or extension of the phase. Specific roles and responsibilities of this team included the following: 1) independent review of the achievement of the Program Goals and Objectives at the end of each Phase, 2) approval of the Gate Review Report to the DOE that was prepared by the PMT, and 3) management of the relationship between Algenol and The Dow Chemical Company.

In 2011, potential non-environmental issues were identified on Dow's Freeport, TX site. As a result, the IBR primary site was moved to Fort Myers, FL. Once the IBR site was moved to Fort Myers, the Dow representatives were replaced on the PMT and IGT.

Subtask A.2 Organism development

Completed July 2011

Biological research at Algenol is focused in three primary areas: strain development, strain characterization, and contamination control. There are also aspects of biological R&D that intersect with cultivation work conducted by the cultivation engineering team, especially regarding development of optimal culturing conditions. Biology researchers are also responsible

for environmental, biochemical, and molecular biology studies in support of regulatory filings for commercial deployment. Biological research takes place at both the Fort Myers and Berlin laboratories. Support for these efforts is provided by Analytical Chemistry and Bioinformatics teams. Although the majority of the work described in this section was funded outside of the DOE IBR grant, we feel that it is worth sharing this information since the strategies for strain development were developed concomitantly and iteratively with the development of the IBR infrastructure and cultivation practices.

Strain Development team

The Strain Development team at Algenol is responsible for creating new cyanobacterial strains that produce ethanol at high rates for an extended duration. Strain development activities have progressed through several phases since the founding of Algenol. Shown below are highlights of past strain development activities, including both the history behind the identification of Algenol's platform production host strain as well as the genetic enhancements that have been conferred to Algenol's ethanol-producing strains.

Early research activities utilizing model cyanobacteria

In order to rapidly gain understanding of ethanol production and how to improve its rate of synthesis in cyanobacteria, and also to build its intellectual property portfolio, Algenol took advantage of model species of cyanobacteria that have been studied for many years in academia, and for which numerous genetic tools and techniques already existed. The first strain utilized was *Synechococcus elongatus* PCC 7942, which was originally transformed to become an ethanol-producing strain at the University of Toronto by John Coleman and Ming-de Deng (Deng M.D. and Coleman J.R. (1999) Ethanol synthesis by genetic engineering in cyanobacteria. Appl. Environ. Microbiol 65: 523-528). This work led to foundational patent rights for Algenol. Another strain that was used extensively at Algenol was *Synechocystis sp.* PCC 6803, a freshwater strain that has been studied throughout the world for many years. Algenol successfully introduced the basic genetics for producing ethanol in this strain, namely the overexpression of genes for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*), and also used this strain to test many additional potential genetic enhancements. Because *Synechococcus elongatus* PCC 7942 and *Synechocystis sp.* PCC 6803 are freshwater strains that do not grow well at high temperatures, Algenol then turned its attention to the identification of strains that were more relevant for commercial operations.

Algenol Biofuels Culture Collection (ABCC) – Algenol scientists worked diligently to establish and expand a culture collection containing over two thousand cyanobacteria, which were acquired by various means, including accessing strains from public and private culture collections as well as actual collection and isolation of strains from various habitats. The strains in the ABCC were then screened for attributes deemed to be essential or beneficial for commercial ethanol production. These attributes included the ability to grow rapidly in salt water, tolerance to high temperature (up to 55°C), and ability to survive for long periods of time in the presence of ethanol. In addition, the morphology and growth habit of the strains in the collection were assessed in order to select those that were most suitable for mass cultivation in Algenol's proprietary photobioreactors (PBRs). Over the ensuing years, the most promising strains were prioritized, leading to eight potential commercial strains that were further studied, including work to establish the ability for several of the strains to produce ethanol. One strain that received significant attention in this regard was ABCC1535, a marine *Synechococcus* strain. Considerable efforts were directed at developing and applying genetic tools for this potential host organism, and very good progress was made in increasing the rate of ethanol production through a variety of mechanisms, most of which were oriented toward improving the ethanol gene cassette, i.e., optimized *pdc* and *adh* genes and the regulatory elements needed to drive

the expression of these genes. Although these advancements with ABCC1535 bolstered Algenol's know-how and intellectual property portfolio, there were a few concerns about this strain (including non-ideal temperature tolerance, genetic instability, and sensitivity to high oxygen levels), that led Algenol to the decision to identify and focus on an even better commercial production host strain. After some additional scoping research, followed by critical examination of the pros and cons of the top candidate strains in the ABCC collection, a truly superior strain emerged, referred to simply as AB1 (Figure A-1).

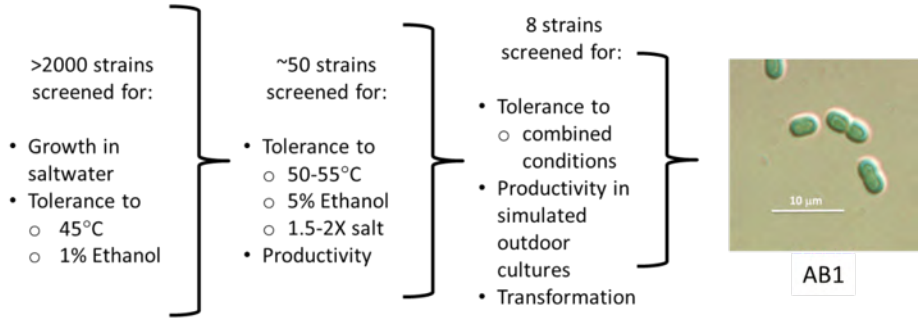


Figure A-1. Host strain identification process.

AB1 is a proprietary cyanobacterial strain that was collected by Algenol scientists in a non-disclosed location. We believe this strain provides significant competitive advantage to Algenol. AB1 grows very well in saline water (including salt water and saline groundwater), has a desirable thermal tolerance range, and is relatively insensitive to high dissolved oxygen levels. The latter point is important since oxygen is a product of photosynthesis and reaches high concentrations in enclosed photobioreactors.

It took a great deal of hard work, perseverance, and creativity to develop the genetic tools and know-how that enabled Algenol to produce ethanologenic strains derived from AB1. As a first step, Algenol sequenced the AB1 genome, providing company scientists with detailed information regarding the genetic makeup of the organism, including the presence and absence of particular biochemical pathways, the preferred genetic code embedded in the DNA, the exact nucleotide sequences of numerous promoters and other regulatory sequences that impact gene expression, the presence and sequences of extrachromosomal elements (i.e., plasmids), etc.

In order to create derivatives of AB1 with high ethanol production rates, it was necessary to develop a genetic transformation system, which enables the introduction and expression of specific genes, such as *pdc* and *adh*. This was not an easy task. In fact, it took a significant and dedicated effort by numerous Algenol scientists to overcome the various hurdles presented by AB1. These hurdles included the need to develop shuttle vectors that were capable of replicating in both *E. coli* and AB1, the identification of suitable selection marker genes, the presence of inherent restriction systems in AB1 that required modification and protection of the introduced DNA, and physical barriers to DNA entry (e.g., robust cell walls surrounded by an exopolysaccharide sheath). Nonetheless, the team was ultimately successful in developing efficient transformation protocols, primarily by the use of conjugation with *E. coli*. We consider our ability to transform this excellent host strain a major accomplishment for the company. The first transformation system developed at Algenol for AB1 utilized an endogenous 6.8 kb plasmid (pAB1A) that was modified in a way that facilitated insertion of heterologous genes, conjugation, and selection of transformants utilizing antibiotic resistance marker genes. See Figure A-2 for a map of the modified pAB1A plasmid (referred to as plasmid TK180) that has been the basis for the generation of many genetically modified strains at Algenol. This transformation system has been used to achieve very high and sustained ethanol production in AB1 derivatives, and continues to be the workhorse transformation system at Algenol.

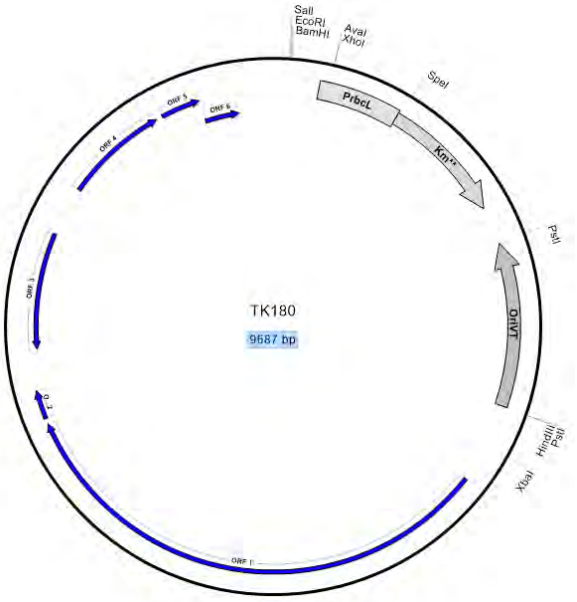


Figure A-2. Map of base shuttle vector TK180 that was generated from endogenous plasmid pAB1A.

Subsequently, Algenol scientists were successful in achieving double crossover homologous recombination in a reliable fashion in AB1; this breakthrough enabled company scientists to make targeted, site-specific gene introductions, knockouts (gene inactivation), and gene replacements, greatly expanding our metabolic and genome engineering capabilities. We have used this system to inactivate a number of genes, including those involved in biochemical processes that divert fixed carbon and energy sources away from ethanol biosynthesis. Homologous recombination has also proved useful for the development of transformation selection systems that do not rely on the use of antibiotic resistance genes, which will likely be important for gaining widespread regulatory agency approval for Algenol's strains and production processes.

Molecular approaches for enhancing ethanol production in cyanobacteria

Efforts to enhance the ethanol production capacity of AB1 via genetic modification have been focused in the following areas:

1. Optimizing the "ethanol cassette" (genes encoding the ethanol biosynthesis pathway)
2. Enhancing the photosynthetic light reactions (capture and conversion of light energy)
3. Maintaining photosynthetic dark reactions (CO2 fixation) at high levels
4. Increasing the conversion of fixed carbon to pyruvate, the precursor for ethanol biosynthesis
5. Decreasing the activity of biochemical pathways that generate biomass rather than ethanol
6. Reducing the impact of genetic mutations that result in lower ethanol productivity
7. Improving competitiveness against contaminating microbes

Items 1-5 in this list are directed primarily at increasing ethanol productivity throughout the entire cultivation cycle, whereas items 6 and 7 are primarily related to increasing the longevity and duration on ethanol production.

1. Optimizing the ethanol cassette – The core element of ethanol production in genetically enhanced cyanobacteria is the expression cassette comprising the introduced transgenes that encode pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*) (Figure A-3). The pyruvate decarboxylase enzyme (PDC) catalyzes the conversion of pyruvate to acetaldehyde, which is subsequently reduced to ethanol in an NADPH-dependent reaction catalyzed by alcohol dehydrogenase (ADH).

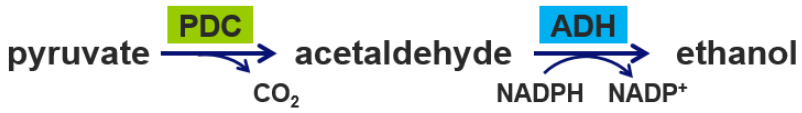


Figure A-3. Ethanol metabolic pathway.

Expression cassettes were produced that had both the *pdc* and *adh* genes in an operon under the control of a single inducible promoter (Figure A-4) or with the *pdc* gene under the control of an inducible promoter and the *adh* gene under the control of a constitutive promoter (Figure A-5).

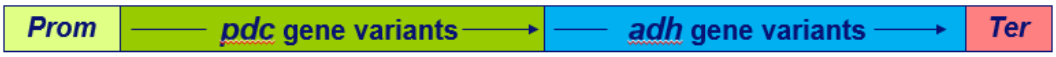


Figure A-4. Single operon ethanol cassette under the control of an inducible promoter.

Figure A-5. Ethanol cassette with a pdc gene under the control of an inducible promoter (Prom1) and an adh gene under the control of a constitutive promoter (Prom2).

A substantial research effort was made to improve the effectiveness of the ethanol cassette. One of the first considerations is which *pdc* and *adh* genes to utilize. Algenol scientists tested a number of different *pdc* and *adh* genes from various sources to identify the best performing candidates; these heterologous genes were further enhanced by altering the DNA sequences to optimize the codon usage. The *pdc* gene that was selected was based on the gene from *Zymomonas mobilis*, an ethanologenic bacterium. Several different versions of this gene were synthesized that incorporated different codon usages, and empirical testing of these variants led Algenol to select a particular variant for use in many versions of the ethanol cassette.

The choice of which *adh* gene to utilize received considerable attention and research effort. A primary consideration in this regard is the overall catalytic efficiency and ability to utilize NADPH as the reductant, but also the affinity of the enzyme for both acetaldehyde (for the desired forward reaction) and ethanol (for the undesired back reaction). Ideally, the ADH should have high affinity for acetaldehyde (*i.e.*, lower K_m) and low affinity (*i.e.*, higher K_m) for ethanol. To this end, *adh* genes from a number of cyanobacteria were identified and screened for activity, followed by analysis of the kinetic properties of the various enzymes in cell-free extracts. The results of these analyses are indicated in Table A-1.

Table A-1. The kinetic properties of various cyanobacterial ADH enzymes determined in crude cell extracts.

Organism (ADH#)	$K_{m(MeCHO)}$ [mM]	$K_{m(EtOH)}$ [mM]	$K_{m(EtOH)}$ / $K_{m(MeCHO)}$
Lyngbya sp. (ADH111)	0.0058 (±0.0011)	0.83 (±0.084)	143
Arthrospira platensis (ADH242)	0.0023 (±0.0005)	2.64 (±0.11)	1056
Microcystis aeruginosa (ADH1520)	0.0978 (±0.0169)	43.3 (±5.4)	443
Cyanothece sp. (ADH553)	0.0756 (±0.0056)	9.33 (±1.39)	123
Synechococcus sp. (ADH971)	0.731 (±0.070)	32.4 (±12.4)	44
Synechococcus sp. (ADH213)	0.783 (±0.086)	67.0 (±16.3)	86
Synechococcus sp. (ADH916)	1.13 (±0.076)	29.3 (±8.5)	26
Chroococcidiopsis sp. (ADH1502)	1.79 (±0.119)	107 (±18)	60
Arthronema africanum (ADH1507)	3.34 (± 0.31)	279 (±66)	84
Chroococcidiopsis sp. (ADH1102)	3.73 (± 0.15)	124 (±24)	33
Cyanobacterium sp. (ADH801)	6.95 (± 0.83)	306 (±49)	44
Synechocystis sp. (synADH)	0.35 (±0.0385)	19 (±3.61)	54

The *Synechocystis* ADH (synADH), which was used for many of the early constructs at Algenol, has fairly desirable K_m values for acetaldehyde and ethanol (0.35 and 19 mM, respectively), but a much better ratio of K_m (acetaldehyde) to K_m (ethanol) was found for the native ADH enzymes from *Lyngbya* (ADH111), *Arthrospira* (ADH242), and *Microcystis* (ADH1520). Additional research was then conducted to clone these genes and introduce them into AB1 under the control of a constitutive promoter; these strains all incorporated the same *pdc* gene. The ethanol productivity observed for several of these strains in 30-day batch LvPBR cultivations, including the control strain transformed with the comparable *synADH*-containing construct (#1578), are shown in Figure A-6.

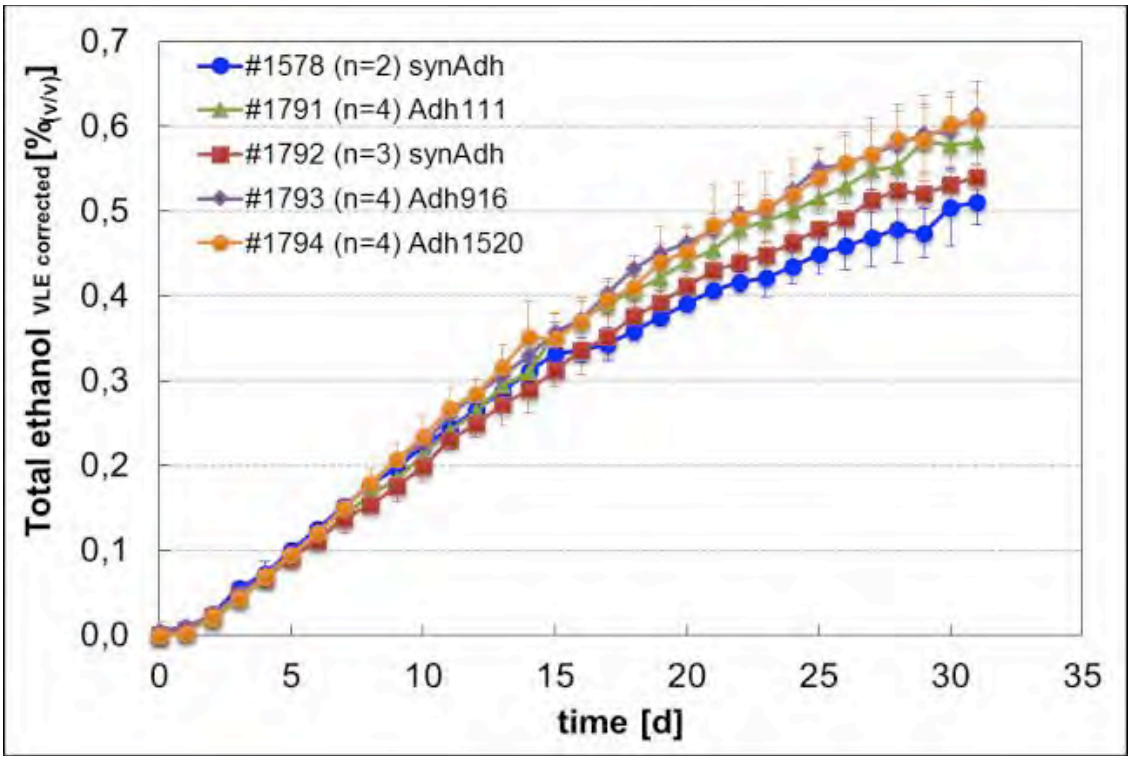


Figure A-6. Ethanol productivity for AB1 strains transformed with ethanologenic plasmids comprising genes that encode synADH, ADH111, ADH916, and ADH1520. Cultivations were conducted in 1-L LvPBRs under standard conditions.

Another important aspect of ethanol cassette optimization was the development of strong, inducible promoters for the expression of the *pdc* gene. The need for inducibility is based on the desire to quickly generate high numbers of cells during the inoculum scale-up phase of the ethanol production process, which is maximal when fixed carbon is not being diverted to ethanol. Thus, it is important that *pdc* promoter "leakiness" (gene expression in the absence of the inducing condition) is minimal during the scale-up phase. It is also important that the promoter maintains high activity throughout the ethanol production cycle. Through significant efforts, the promoter from a native AB1 gene involved in nitrogen metabolism (i.e., nitrite reductase, designated as *nirA*) was identified as a strong promoter for driving the expression of the *pdc* gene; this promoter is repressed in the presence of ammonium and induced in the presence of nitrate, enabling a relatively straightforward induction system wherein ammonium is provided to the cells during the scale-up phase and nitrate is provided at the onset of the ethanol production phase.

Algenol also successfully identified several promoters that are induced in the presence of metal ions (e.g., copper, zinc, nickel) and have successfully applied these promoters for ethanol production and related pathways as well. These promoters were identified through the use of global transcriptomics experiments to analyze RNA isolated from AB1 cultures exposed to various levels of metal ions. Quantitative RT-PCR (qRT-PCR) was then performed on the identified promoters in order to confirm the level of inducibility at various times after addition of the inducing metal. Examples of the results from such analyses are indicated in Figure A-7 (for zinc-induced genes) and Figure A-8 (for copper-induced genes).

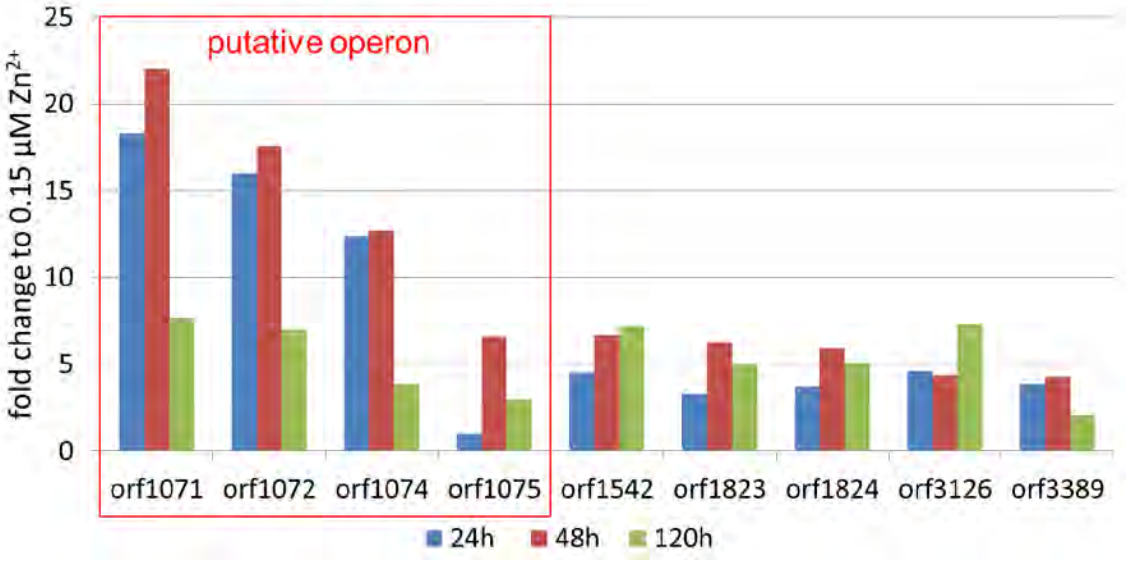


Figure A-7. qRT-PCR analysis of selected zinc-inducible genes of AB1 treated with 10 µM Zn^{2+}. AB1 was grown for two days with reduced metal ion concentrations before addition of 10 µM Zn^{2+}. RNA was extracted 24, 48 and 120 h after zinc addition, and qRT-PCR was performed using orf0132 as housekeeping gene. The fold change was calculated relative to the untreated control cultures.

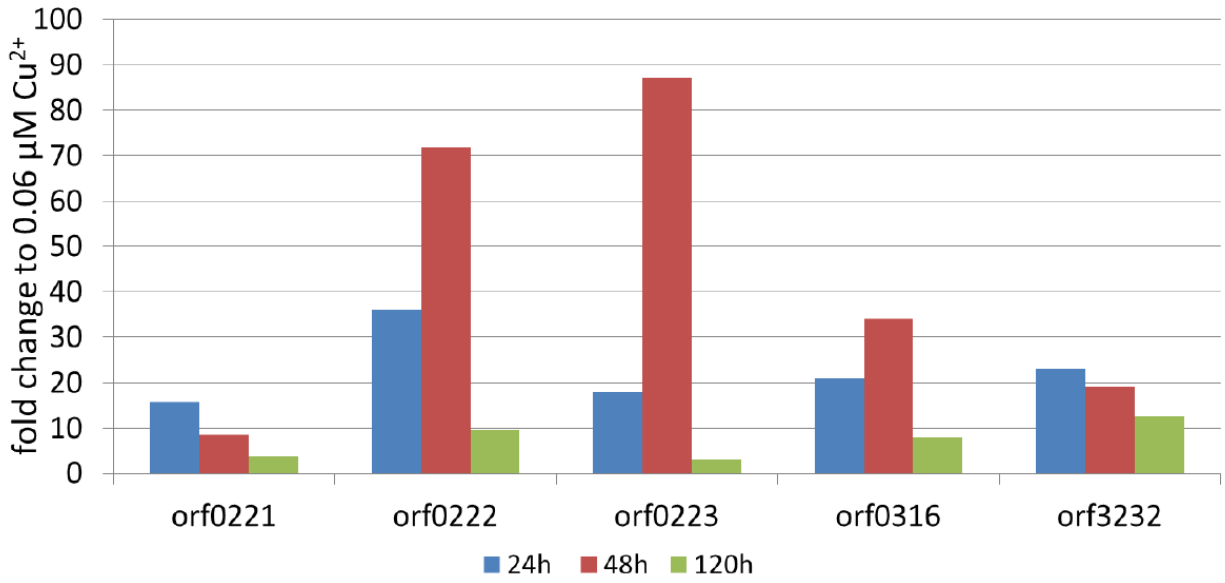


Figure A-8. qRT-PCR analysis of selected copper-inducible genes of AB1 treated with 3 µM Cu^{2+}. AB1 was grown for two days with reduced metal ion concentrations before addition of 3 µM Cu^{2+}. RNA was extracted 24, 48 and 120 h after copper addition, and qRT-PCR was performed using orf0132 as housekeeping gene. The fold change was calculated relative to the untreated control cultures.

Table A-2 shows a list of the differentially regulated genes from AB1 whose promoters were initially prioritized for further evaluation.

Table A-2. Prioritized metal-inducible genes identified from transcriptomics studies. The description refers to the annotation of the protein coding sequence (ORF) based on the best hit in a BLAST bioinformatics analysis.

ORF No.	Description	Inducer
0128	Hypothetical protein	Ni
1486	Putative Ni-containing Superoxide Dismutase	Ni
3621	Hypothetical protein	Ni
1071	Mn transporter	Zn
1542	Hypothetical protein	Zn
1824	No hits found	Zn
3126	Metallothionein	Zn
0221	Copper resistance protein	Cu
0316	Hypothetical protein	Cu
3232	Cation transporting ATPase	Cu
3749	Hypothetical protein	Co

Of particular interest were promoters that could be induced in a dose-dependent manner. Figure A-9 shows an example of increasing PDC activity due to increasing concentrations of copper when three different promoters were included in the ethanol cassette to drive *pdc* expression.

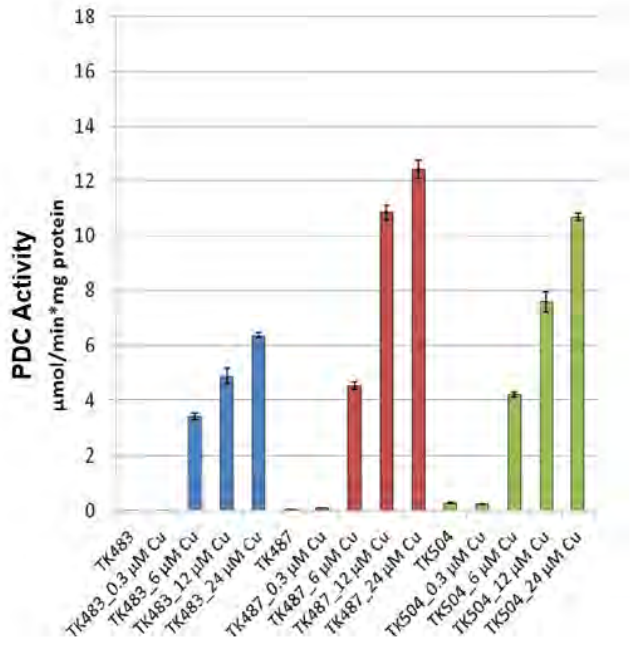


Figure A-9. PDC activities measured in AB1 cells transformed with constructs that utilize the copper-inducible promoters for orf221 (TK483), orf316 (TK487), and orf223 (TK504). Cultures were treated with different levels of Cu^{2+} as indicated in the figure.

To further enhance the effectiveness of these inducible promoters, site-specific modifications to the DNA sequence were made to the ribosome binding site (RBS) and the TATA (Pribnow) box found ~10 bp upstream of the transcription initiation site; these changes were intended to increase the general strength of the promoter for driving gene transcription. In addition, regions of the promoter that were known or hypothesized to be involved in the binding of various transcription factors were modified in order to alter the "tightness" or "leakiness" of the promoter under non-induced conditions. Examples of such modifications that were made to the *nirA* promoter are shown in Figure A-10. In this case, in addition to modifications to the RBS and TATA box regions, the NtcA and NtcB transcription factor binding sites (responsible for controlling promoter activity in response to ammonium and nitrate levels) were altered. In some cases, the overall activity of the promoter was enhanced significantly (generally a desirable outcome), although in some cases this also resulted in higher activity in the uninduced state (generally an undesirable outcome). Therefore, it is important to test empirically the overall effect of such promoter modifications on actual ethanol productivity and culture health.

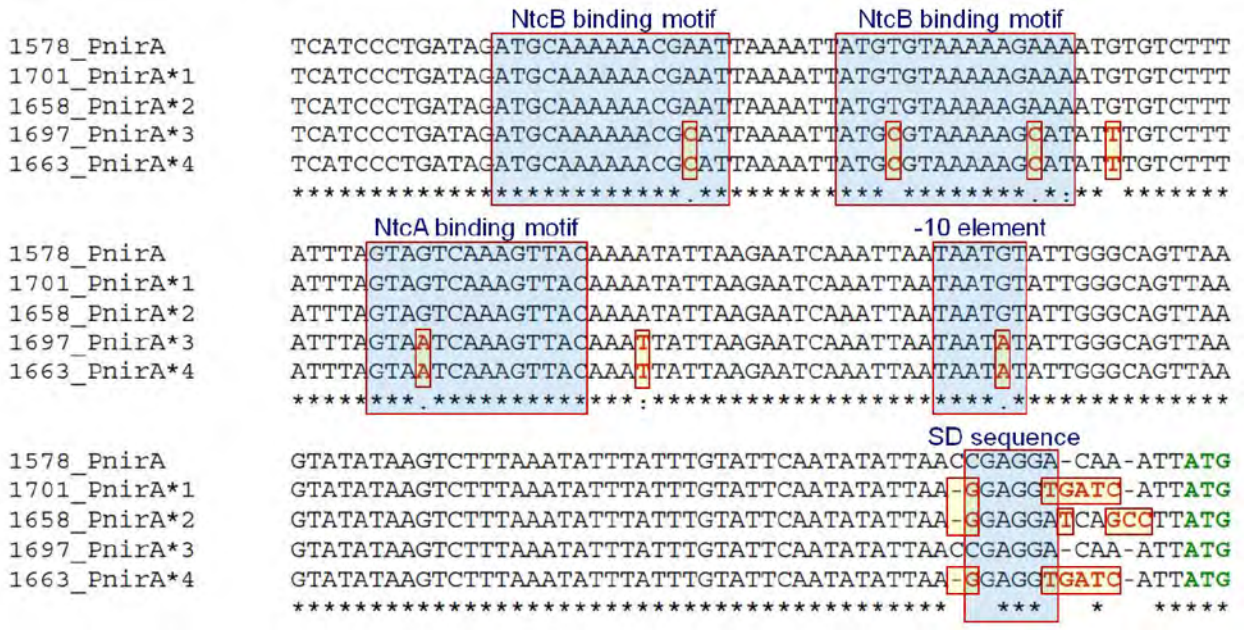


Figure A-10. Alignment of the nirA promoter sequences containing site-directed point mutations generated within both NtcB-binding motifs, the NtcA binding motif, the TATA box (also called -10 region) as well as within the RBS (Shine-Dalgarno, or SD) sequence.

The results of using these modified *nirA* promoters to drive expression of the *pdc* gene in AB1 are shown in Figure A-11, which shows the uninduced and induced PDC activities in ethanologenic AB1 strains.

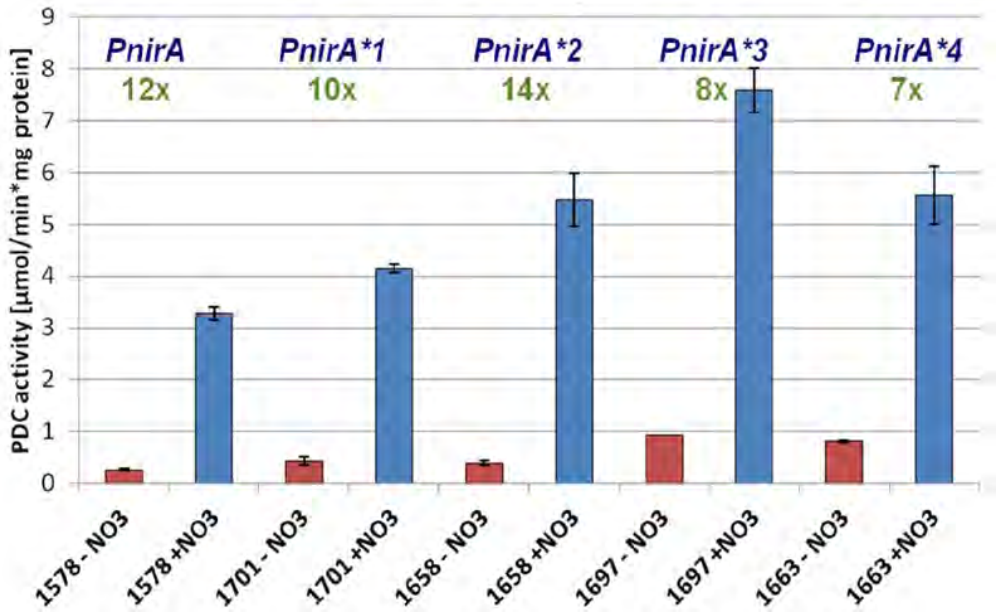


Figure A-11. PDC activity in AB1 cells transformed with pdc driven by the native nirA and various derivatives in the presence and absence of the inducer nitrate.

PDC activity has a large impact on carbon partitioning to ethanol *vs* biomass, which generally translates to higher overall ethanol productivity. Thus, the increased activity of the improved *nirA* promoter variants had a significant impact on both of these parameters. The impact of the higher activity of the *nirA*2* promoter on carbon partitioning to ethanol is shown in Figure A-12.

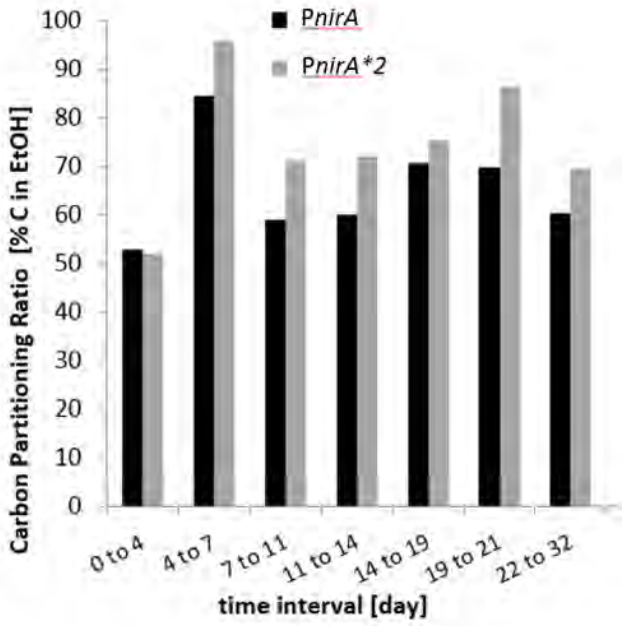


*Figure A-12. Carbon partitioning into ethanol at various culture stages in an AB1 strain using the native nirA promoter (PnirA) to drive pdc expression vs the modified nirA*2 promoter (PnirA*2).*

2. Enhancing photosynthetic light reactions – The energy and fixed carbon used for ethanol production are supplied by photosynthesis, which can be broken down into the "light reactions" and the "dark reactions." The "light reactions" result in the conversion of the energy inherent in

photons to chemical potential energy in the form of NADPH and ATP. These products are subsequently used to reduce CO_2 to organic carbon molecules *via* the Calvin-Benson-Bassham (CBB) cycle (the "dark reactions") as well as for other biochemical reactions in the cell, including the use of NADPH in the ADH reaction to form ethanol. NADPH is generated by the reduction of $NADP^+$ with electrons that flow in a linear fashion (linear electron transport, or LET) from the water splitting reaction of Photosystem II (PSII) through to the high energy state of Photosystem I (PSI). ATP is formed *via* the activity of an ATPase energized by a proton gradient established across the thylakoid membrane as a result of LET between PSII and PSI. ATP can also be generated by cyclic electron transport (CET), wherein high energy electrons activated through PSI are fed back into the electron transport chain between PSII and PSI, contributing to the proton gradient across the thylakoid membrane, but not resulting in $NADP^+$ reduction.

The balance of LET and CET can have a significant impact on the ratio of ATP to NADPH within cells; this is important because the quantities of these compounds necessary for biomass generation *versus* ethanol biosynthesis are different. This balance is regulated in cells through several mechanisms, and can be impacted by the amount of ADP and $NADP^+$ available for conversion to ATP and NADPH, the ratio of PSI complexes to PSII complexes, the redox state of the photosystems and electron transport components, etc. The availability of ADP and $NADP^+$ for phosphorylation and reduction can be impacted by futile cycles involving ATP hydrolysis and/or NADPH oxidation in addition to normal biosynthetic processes that utilize these compounds. Algenol scientists produced a number of different strains that were modified in ways anticipated to favor LET *versus* CET and/or that were designed to improve the ratio of ATP to NADPH in a way that would hopefully increase the rate of ethanol biosynthesis.

The question has often arisen about whether photosynthetically-generated NADPH levels may be limiting the rate of ethanol production. One line of evidence argues against this, however — feeding illuminated ethanologenic cells with acetaldehyde resulted in significantly higher rates of ethanol production as compared to cells fed only with CO_2. Although these results may indicate fixed carbon substrate limitation, they suggest that NADPH is not limiting for ethanol production under the conditions tested. It should be recognized, however, that feeding high levels of acetaldehyde could have pleiotropic effects that make data interpretation somewhat difficult.

Several experiments with various ethanologenic strains indicated a clear reduction in net photosynthetic capacity (P_{max}) of cells as the culture aged; this reduction paralleled reductions in ethanol productivity. P_{max} is typically determined for cells removed from a culture by measuring O_2 evolution with an oxygen electrode at various light intensities in the presence of saturating amounts of CO_2, and thus reflects the point at which the rate-limiting step for the entire photosynthetic process is no longer attributable simply to the irradiance level. Although we cannot be certain of the extrapolation of these results to actual net photosynthesis rates in an operating PBR in which the cells are maintained at low average (but somewhat fluctuating) light levels, they do suggest that lower photosynthetic carbon fixation rates are a significant factor in the downturn in ethanol productivity. It is also worth noting that the light saturation constant (E_k), which is a measure of the irradiance level necessary to achieve P_{max}, also decreases with culture age; this can be attributed to adaptation of the cells to lower average light levels per cell due to increasing culture density.

The specific reason(s) behind the observed reduction in P_{max} over time are not completely clear. A reduction in downstream carbon fixation can be the outcome of acclimation to low light levels in that it is well established that irradiance levels affect the expression of genes involved in the dark reactions of photosynthesis as well as the catalytic function of the encoded enzymes themselves (mediated in many cases through redox conditions within the cell).

An experimental approach to maximize photosynthetic light utilization in algal cultures that has received some attention in the literature is called the Truncated Light-Harvesting Antenna (TLA) concept. In this strategy, the cellular concentrations of photosynthetic pigments (chlorophyll and/or phycobiliproteins) are artificially reduced by genetic manipulation to levels below that usually obtained in growing cultures of increasing density. This provides two benefits for improved light utilization: 1) there is a reduction in self-shading such that light penetration into the PBRs is improved and more cells are exposed to some degree of illumination, and 2) at the cellular level, the reduced capacity for light absorption by individual cells with smaller light harvesting arrays reduces the potential for over-excitation (at the PBR surface) and the resulting loss of energy through non-productive processes (fluorescence) or photo-oxidative damage to electron transport components. In this modified light environment, due to lowered total pigments, the cell signaling processes that would normally reduce P_{max} and E_k as a result of increasing cell densities and diminishing light levels may be delayed, and the culture may maintain a higher level of photosynthetic capacity over a longer period of time in batch culture growth relative to cultures with normal pigment levels. To test this premise, inducible knockdown and genomic knockout strategies were used to modify the expression of the phycocyanin (PC) genes *cpcA* (deleted) or *cpcBA* (operon expression reduction) in ethanologenic AB1 strains. This resulted in cell lines with varying levels of PC, the primary light harvesting component of cyanobacteria. The variation in total PC content obtained in these lines relative to control cultures is shown in Figure A-13. Further testing of these cells is underway.

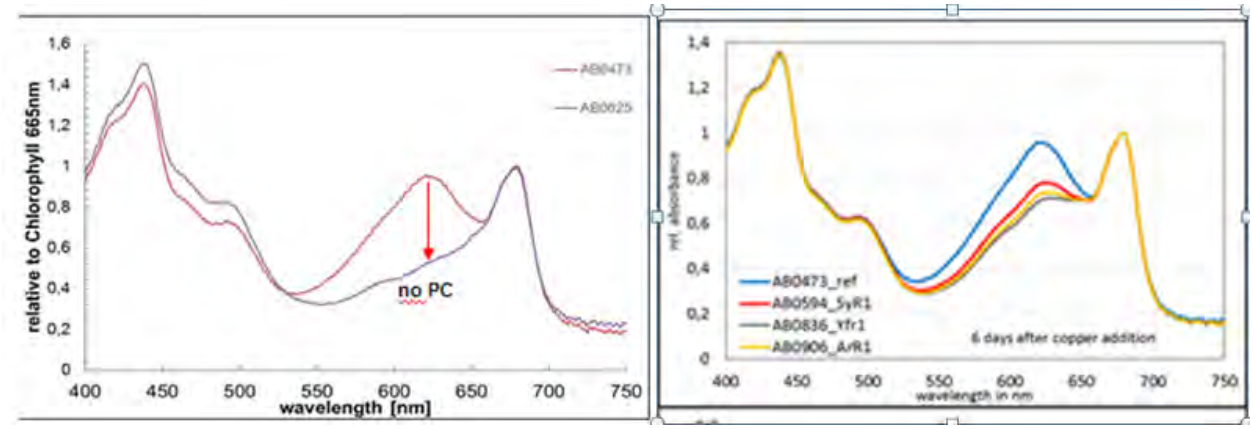


Figure A-13. Impact of cpcA deletion (left panel) and varying levels of reduced cpcBA expression obtained by antisense RNA expression (right panel) on PC levels of ethanologenic AB1 lines.

A phenomenological productivity model developed at Algenol, which is based on data from numerous experiments performed both indoors and outdoors, suggests that most of the downturn in ethanol productivity in long duration cultures can be explained by light limitation. In this model, once essentially all incident light is absorbed by the cultures, the rate of carbon fixation for a given volume of the culture is constant, but the increasing number of cells in that volume requires that a higher proportion of the fixed carbon must be utilized for respiration in order to satisfy energy-requiring cell maintenance needs (e.g., protein and membrane turnover, photosystem repair, etc.). This model assumes a constant quantum yield (α) for total carbon fixation over the growth cycle and that respiration is a direct and linear function of cell number (estimated in these models as OD_{750}), such that the amount of fixed carbon utilized for respiration becomes a larger and larger proportion of the total carbon fixed. Some limited data on α and nighttime respiration in different growth modes support these assumptions for the experiments examined, although more data across the growth cycle would be useful for further validation. In some cases, it appears that a decline in α may occur over the course of long cultivations, which is not surprising considering the different metabolic states a culture

progresses through during a typical cultivation. The model also includes the effects of decreasing E_k with culture age, also a consequence of culture densification, as discussed above. This reduction in E_k is clearly a driver of the reduced productivity observed over time. Additional details of this productivity model are provided in the Production Model section.

Cyanobacteria are rather unique in that the respiratory electron transport (RET) and photosynthetic electron transport (PET) chains share certain components. Electrons from succinate, a product of the tricarboxylic acid cycle, are fed into the plastoquinone (PQ) pool of the PET chain by the activity of succinate dehydrogenase (SDH). The flux of electrons through the electron transport components originating from respiration *versus* photosynthesis is determined primarily by the availability of light, and involves complex regulation based on the redox status of the PQ pool, and can include physical repositioning of the SDH and RET/PET components within the thylakoid membrane. The contribution of electrons from succinate to the PQ pool during periods of illumination is not well understood, as it is rather difficult to separate RET from PET.

Photooxidative processes that generate reactive oxygen species (ROS) or that reduce O_2 back to H_2O can occur under certain conditions such as high light, low temperature, or low CO_2 levels, all of which can result in over-reduction of the PET chain due to an excess of NADPH (and corresponding lack of $NADP^+$ to serve as an electron acceptor from reduced ferredoxin produced through PSI activity). This can result in the production of superoxide from PSI and singlet oxygen from PSII, both of which can cause oxidative damage to the photosystems and other cellular components. Although the reduction of O_2 back to H_2O (the "water-water" cycle) does not cause cellular damage, it still results in the loss of photosystem-generated reducing power that could otherwise be used to fix CO_2. Photosystem-based O_2 reduction in cyanobacteria is mediated by flavodiiron proteins (encoded by *flv1* and *flv3*), which combine to form a functional heterodimer. It was hypothesized that elimination of this protein by insertional inactivation of the *flv1* and/or *flv3* genes may increase light utilization efficiency.

3. Maintaining photosynthetic dark reactions (CO_2 fixation) at high levels – In the photosynthetic "dark reactions," ATP and NADPH are utilized in the Calvin-Benson-Bassham (CBB) cycle to fix CO_2 to organic compounds. The initial step in the CBB cycle is the carboxylation of ribulose-1,5-bisphosphate (RuBP) to form two molecules of 3-phosphoglycerate (3-PGA), catalyzed by the enzyme RuBP carboxylase/oxygenase ("Rubisco"). In cyanobacteria, Rubisco is found within a structured microcompartment referred to as a carboxysome, which is composed of shell proteins that form uniform hexagonal faces with defined pore structures; these pores appear to modulate the entry of RuBP and HCO_3^- and the exit of 3-PGA. Importantly, carbonic anhydrase, which catalyzes the conversion of HCO_3^- to CO_2, is also found within the carboxysomes, enabling a greatly elevated ratio of CO_2 to O_2 in the vicinity of Rubisco, thus minimizing wasteful photorespiratory processes that can occur due to the oxygenase side reaction of Rubisco.

After exiting the carboxysome, the 3-PGA is metabolized *via* central carbon metabolism pathways, including glycolytic conversion to pyruvate (followed by conversion to ethanol or other biochemicals), the CBB cycle for regeneration of the CO_2 acceptor RuBP, and gluconeogenesis to form six-carbon sugar-phosphates that are further converted into a large variety of compounds and biochemical intermediates. In order to regenerate RuBP for additional rounds of CO_2 fixation by Rubisco, a portion of the 3-PGA must first be phosphorylated to 1,3-PGA *via* the activity of phosphoglycerate kinase (PGK) and then reduced to glyceraldehyde-3-P (GAP) *via* the activity of GAP dehydrogenase (GAPDH); this is followed by a complex series of enzymatic steps in the CBB cycle in order to regenerate RuBP. The final step in RuBP regeneration is catalyzed by phosphoribulose kinase (PRK). Each mole of 3-PGA produced from the CBB cycle requires 9 moles of ATP and 6 moles of NADPH, when taking into account the regeneration of

RuBP. It is important to note that Rubisco could become limited for RuBP if excessive amounts of 3-PGA were directed into ethanol; such a scenario would result in a reduction of photosynthetic rates.

Although the rate of photosynthetic carbon fixation is clearly dependent on the rate of ATP and NADPH generation from the light reactions, it can also be impacted by the activities of the enzymes of the CBB cycle. Rubisco has been demonstrated in many photosynthetic cells to be under significant regulatory control at the transcriptional and post-translational levels. It is possible that a reduced level of carboxylation capacity limits the availability of newly fixed carbon for ethanol synthesis and results in the decline in ethanol production with culture age. To mitigate this potential loss, ethanologenic lines were generated with plasmid-based constructs containing the native Rubisco operon (encompassing *rbcLXS*, which encodes the large and small subunits and the assembly chaperone) under inducible and constitutive promoters. These overexpression constructs also included modifications (site directed DNA sequence changes to both large and small subunits) designed to enhance catalytic activity. Characterization of these ethanologenic lines during batch cultivation under standard indoor production conditions confirmed Rubisco overexpression at the mRNA and protein level for two specific strains relative to the unmodified lines (see Figure A-14). These strains exhibited between 20 and 40% higher Rubisco activity, which was associated with higher maximal photosynthetic rates (P_{max}). The data also showed a 20-30% increase in biomass productivity in terms of increased culture density (as measured by OD_{750}) and increased dry weight (DW). Culture ethanol levels were only marginally higher relative to unmodified ethanologenic lines, suggesting partitioning of the increased fixed carbon is into metabolic compartments not accessible for mobilization into the ethanol synthesis pathway.

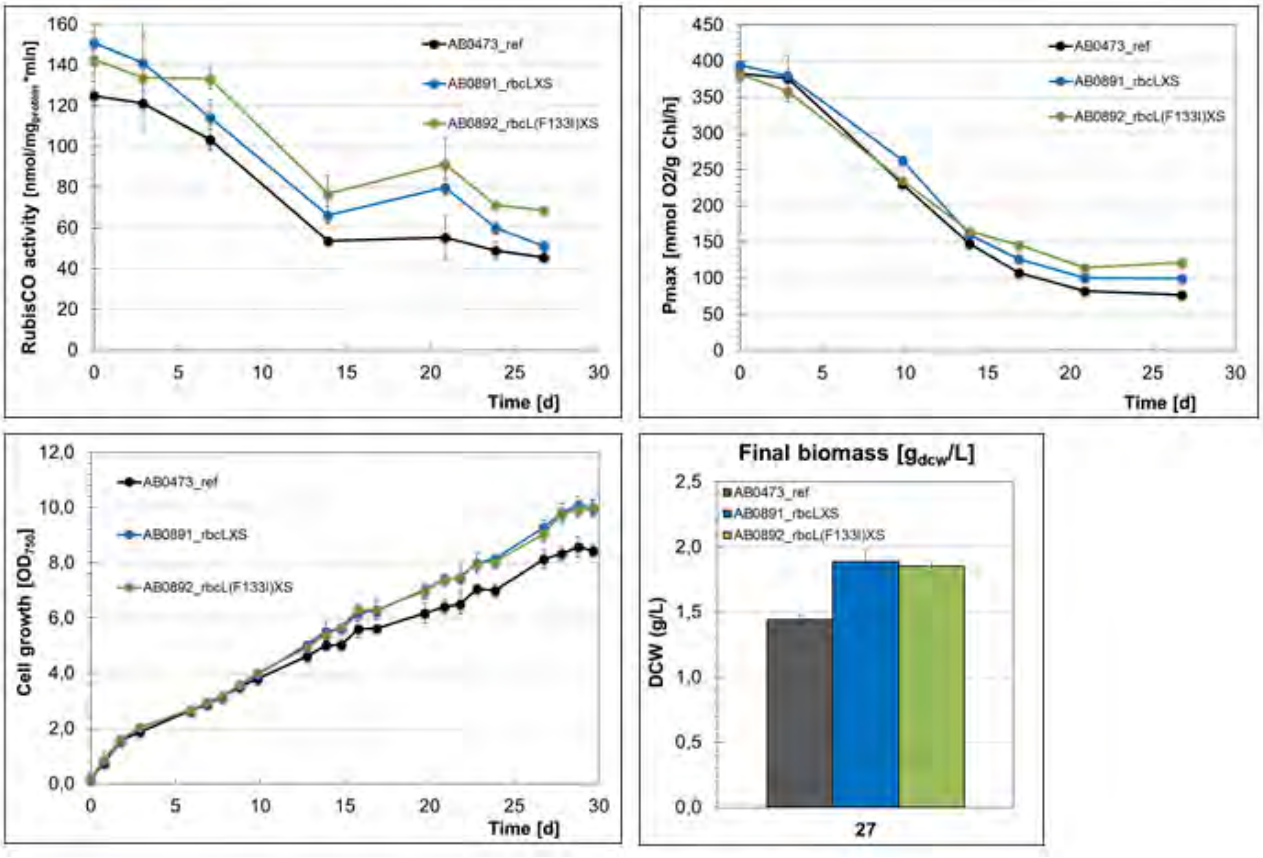


Figure A-14. Impact of Rubisco overexpression on catalytic activity, P_{max}, cell growth and final biomass achieved in batch culture.

Another factor that could potentially limit maximal rates of ethanol biosynthesis is the availability of inorganic carbon (C_i) to cells in dense cultures. Growth room experiments with LvPBRs using various combinations of CO_2 gassing flow rates, alkalinity, and irradiance indicated that sufficient CO_2 was generally available to the cells under the indoor culture conditions, but for outdoor cultures variable mixing rates and increased culture viscosity may potentially limit the availability C_i to individual cells.

4. Increasing the conversion of fixed carbon to pyruvate – The conversion of the CBB cycle product 3-PGA to pyruvate is the critical part of central carbon metabolism required to generate the substrate needed for ethanol production (Figure A-15). Several enzymes are involved in this process, representing the lower segment of the glycolysis pathway. The reaction steps are indicated below:

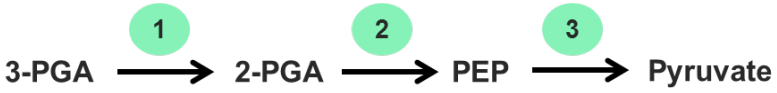


Figure A-15. 3-PGA metabolism to pyruvate.

Enzymes:	Compounds:
Phosphoglycerate mutase (PGM)	3-PGA = 3-phosphoglycerate
Enolase	2-PGA = 2-phosphoglycerate
Pyruvate kinase (PK)	PEP = phosphoenolpyruvate

Since pyruvate is the substrate for the PDC enzyme, a reduction in the activity of PGM, enolase, or PK could, in principal, lower the rate of ethanol production. Biomass could continue to increase, however, since 3-PGA can be converted to GAP and undergo gluconeogenesis to ultimately form glucose-6-P, which can then be further metabolized into a variety of biomass components. In addition, if blockage of the last enzymatic step in this sequence (pyruvate kinase) were to occur, PEP carboxylase (PEPC) could catalyze the formation of oxaloacetate, which would also potentially contribute to biomass formation.

5. Decreasing activity of biochemical pathways that generate biomass rather than ethanol – For purposes of this discussion, biomass is defined as any compound or material comprising stably fixed carbon other than ethanol. Carbohydrates, proteins, lipids, and other biochemicals, including materials that are released to the medium as dissolved organic compounds ("DOC"), are included in this category.

Multiple studies with ethanologenic AB1 strains have shown that the rate of ethanol production is reduced more substantially (and earlier) than the rate of biomass production, resulting in a lowering in the fundamental carbon branching ratio for ethanol biosynthesis (defined as the rate of carbon fixation into ethanol divided by the total carbon fixation rate). A change in carbon partitioning would result from changes in the relative activities at key branch points in central carbon metabolism. The branch point that has received the most attention involves pyruvate metabolism, such that the amount of fixed carbon partitioned into ethanol *versus* biomass would be reduced if the *in vivo* activity of PDC is lowered relative to the activity of enzymes involved in biomass generation (e.g., pyruvate dehydrogenase [PDH], phosphoenolpyruvate carboxylase [PEPC], key enzymes of gluconeogenesis and storage compound synthesis, etc.). These competing pathways are illustrated in Figure A-16 and described in more detail in the following paragraphs.

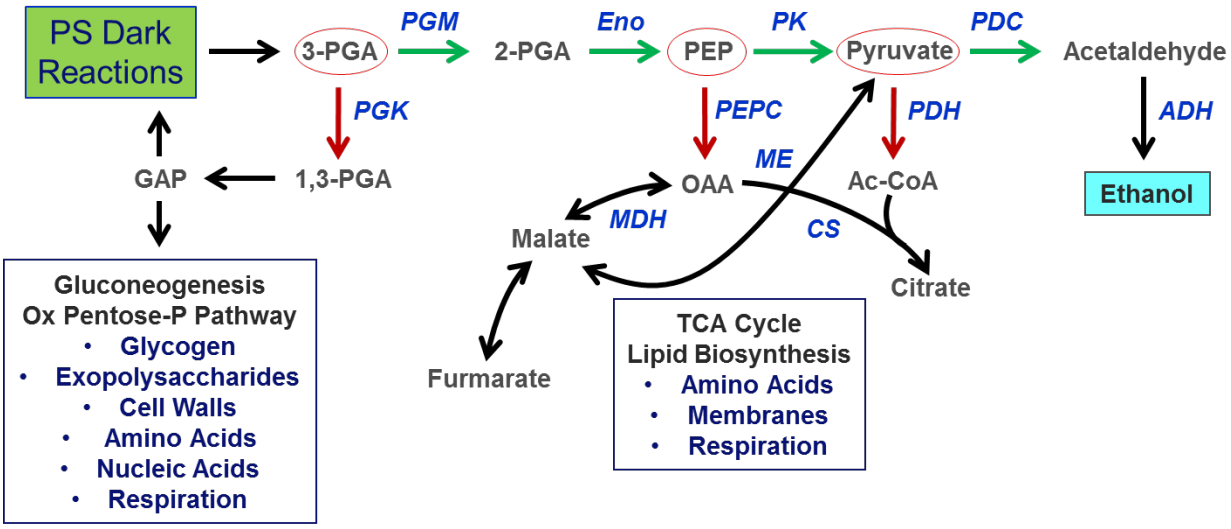


Figure A-16. Branch points in carbon metabolism that can lead to biomass production instead of ethanol production.

– Metabolism of pyruvate through the tricarboxylic acid (TCA) cycle: The relative *in vivo* activities of PDH and PDC would be expected to play a significant role in determining the partitioning of pyruvate to form either biomass or ethanol. Acetyl-CoA, the product of the PDH reaction, enters into the TCA cycle by combining with oxaloacetate (OAA) to form citrate, which is then metabolized by the various enzymes of the TCA cycle to produce energy (ATP and NAD(P)H) and biochemical intermediates that are further converted to various amino acids and

other biomass components. Acetyl-CoA is also the building block for lipids, which are primary components of the cell's membrane systems.

– *Gluconeogenesis:* Gluconeogenesis, the reverse of glycolysis, leads to the synthesis of hexose-phosphates that can be further metabolized into polysaccharides, including storage carbohydrates such as glycogen and structural polysaccharides such as exopolysaccharides (EPS) and cell wall/sheath polysaccharides, or can be metabolized via the oxidative pentose phosphate (OPP) pathway to generate NADPH as well as additional biochemical intermediates.

– *Storage carbohydrate formation:* The primary form of stored carbon in cyanobacteria is usually glycogen or a semi-amylopectin type of glucan; these are polysaccharides that are formed from glucose units linked together by α-1,4 and α-1,6 bonds. Storage glucan levels have been measured in wild-type and ethanologenic AB1 cultures growing in lab PBRs at relatively low light levels (125 $\mu E \cdot m^{-2} \cdot sec^{-1}$ from one side; ~35-50% of the average outdoor light for VIPERs, depending on the PBR spacing). Preliminary results suggest that the amount of glycogen formed over the course of a day was equivalent to ~10% of the amount of ethanol produced per day, and that ethanologenic lines exhibited lower levels of glycogen prior to the onset of illumination (end of dark phase) in a typical 12/12 hour photoperiod, and the accumulation of elevated levels of glycogen (relative to non-ethanologenic lines) at the end of the light phase. Attempts to increase C fixation in ethanologenic lines by enhancing levels of glycogen accumulated during the day were attempted through various means. It is known that glycogen catabolism over the night period provides respiratory energy for cell maintenance and repair, so it is not clear how much of the carbon that is fixed into glycogen could be redirected into ethanol before deleterious effects would be observed.

– *Oxidative Pentose Phosphate (OPP) Pathway:* Conversion of glucose-6-P, produced through the gluconeogenesis reactions or through glycogen catabolism, to 6-phosphogluconolactone and then 6-phosphogluconate, represents the initial steps of the OPP pathway. This pathway is important for producing energy in the form of NADPH as well as several organic molecules that are used for the synthesis of a variety of compounds, including certain amino acids, nucleic acids, and sugars used for cell wall and exopolysaccharide production. It is important to note that in cyanobacteria, eight enzymes are common to the OPP pathway and the CBB cycle. The 6-phosphogluconate is decarboxylated to ribulose-5-P, which after phosphorylation to RuBP becomes the substrate for Rubisco. Alternatively, the ribulose-5-P undergoes additional rearrangements through other enzymes to produce a number of other biochemical intermediates. This overlap between the OPP and CBB pathways means that the relative activities of key enzymes can have a large impact on the overall photosynthetic carbon fixation rate as well as the production of intermediates that are key to continued cell growth and biomass accumulation. It is worth considering whether an enhancement of the initial reactions of the OPP pathway could lead to higher rates of photosynthesis and a desirable reduction in the ATP:NADPH ratio after the point in the growth cycle where the ethanol synthesis rate declines.

– *Compounds secreted to the medium:* The production and release of dissolved organic carbon (DOC) represents a significant amount of fixed carbon that could potentially be redirected into ethanol. A full analysis of the DOC found in the culture medium of AB1-based strains has not been completed, although there are clearly significant amounts of extracellular polysaccharides present, based on the increased viscosity of the culture medium of late-stage cultures. In order to determine whether the DOC present in a typical outdoor cultivation would create problems with downstream ethanol processing via the VCSS unit, the Complex Carbohydrate Center at the University of Georgia was contracted to perform an analysis of polysaccharides isolated from culture medium of a late stage AB1 culture. This study did not include a specific analysis of the attached exopolysaccharide sheath, although such data would

be informative; such analyses may help to identify target genes for limiting the amount of DOC produced.

6. Reducing the impact of genetic mutations that result in lower ethanol productivity – Observations made at Algenol with several cyanobacterial species have clearly indicated that the introduction of the ethanol cassette and the resulting production of acetaldehyde and ethanol create a stressful situation for the host cells. Furthermore, cell division rates for ethanol-producing cyanobacteria are significantly lower than for wild-type or non-induced cells, as well as for cells that have lost the ability to form ethanol due to mutational inactivation of the ethanol cassette genes; this is simply because so much carbon is channeled into ethanol rather than cell biomass (i.e., more cells) during ethanol production. This selective growth advantage for non-producing cells therefore favors proliferation of mutated cells ("revertants") under culture conditions that promote cell division, such as dilute cultures with high light and nutrient availability. Even in the absence of high growth rates, mutations can accumulate in the multiple copies of the ethanol cassette present (both plasmid- and chromosome-localized) because of stress-induced mutagenic processes in cells, including transposon mobilization, an increase in the activity of error-prone DNA and RNA polymerases, and suppression of DNA repair systems.

Population analyses (conducted by assessing variation in colony size and ethanol synthesis capacity of isolated clones) have been used to assess inoculum quality and to understand population dynamics of producing and revertant (non-producing) cells; these analyses have clearly shown the benefits of using inocula with very low levels of revertants, which is best achieved by very tight repression of the expression of the ethanol gene cassette (specifically the *pdc* gene). Results obtained from a large number of laboratory and outdoor cultivations of AB0015 have shown a low percentage (<5%) of revertants in cultures in which the number of generations after inoculation is less than 15. Therefore, mutation events do not result in a significant loss in the ethanol production capacity of AB0015 cultures grown under outdoor conditions after 30 - 45 days of batch cultivation. Consequently, simple batch-mode operation of a commercial production facility would not likely be impacted by reversion events, assuming that high quality inoculum cultures are used. However, population analyses of cultures subjected to repeated dilutions and re-growth indicate rapid increases in the proportion of revertants, and thus improved genetic stability of ethanol-producing cells would be an absolute requirement for semi-continuous culturing in commercial operations, which is currently the expected mode of operation.

Sequence analysis of individual revertant clones has provided information on the types of mutations that occur most frequently, and bioinformatics analysis of the AB1 genome sequence and transcriptomics data of ethanologenic derivatives have identified the primary mechanisms responsible for mutation generation and genetic instability in this strain: insertions of endogenous transposons into the promoter and coding sequence of the *pdc* gene were found to be responsible for a significant proportion of the reversion events. Numerous transposases have been identified in the AB1 genome, but most of the revertants can be accounted for by the activity of five primary transposases. Point mutations and small deletions within the *pdc* expression cassette have also been observed, but were a much smaller proportion of inactivating events relative to transposon insertions.

Algenol scientists have developed a novel method to limit ethanol production losses due to genetic reversion. The method takes advantage of the fact that the majority of the mutation events that lead to loss in ethanol production involve large insertions (i.e., transposons) or deletions in the *pdc* promoter or coding region, such that transcription of the full-length *pdc* gene is rendered impossible. As described in International Patent Appl. No. PCT/US2015/000210, an "essential operon" is created in which a gene that must be expressed for the cell to live (an "essential gene") is inserted directly downstream of the *pdc* gene (as a member of a two-gene

operon). If the essential gene is not expressed because the promoter or *pdc* gene is mutated, then the cells die and therefore are not able to take over the culture. A number of different essential genes can be used in this strategy, but Algenol placed most emphasis on the use of the *nirA* (nitrite reductase) gene. In this case, it was first necessary to inactivate the native *nirA* gene in the host cyanobacterium (typically AB1 and derivatives). The effectiveness of using this strategy can be clearly seen in Figure A-17, which compares the growth and ethanol productivity of an ethanologenic cell line that utilizes the *nirA* essential operon strategy with a control strain that does not include an essential operon after several culture dilutions. Figure A-18 indicates the number of revertant, non-ethanologenic cells in these cultures at various points in the cultivation.

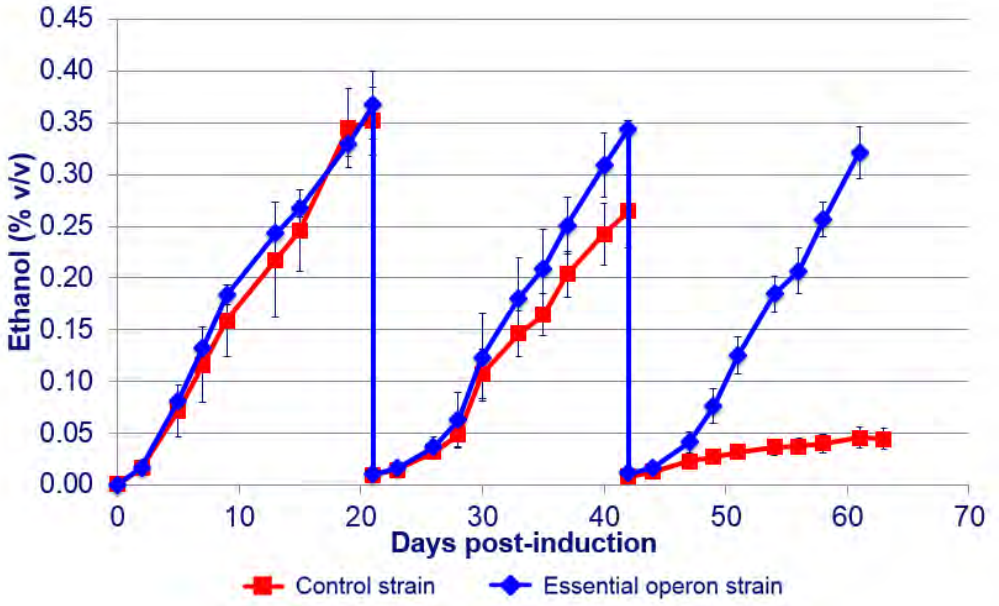


Figure A-17. Ethanol productivity of cultures after low density inoculation and two dilution cycles (20-fold dilutions into fresh medium).

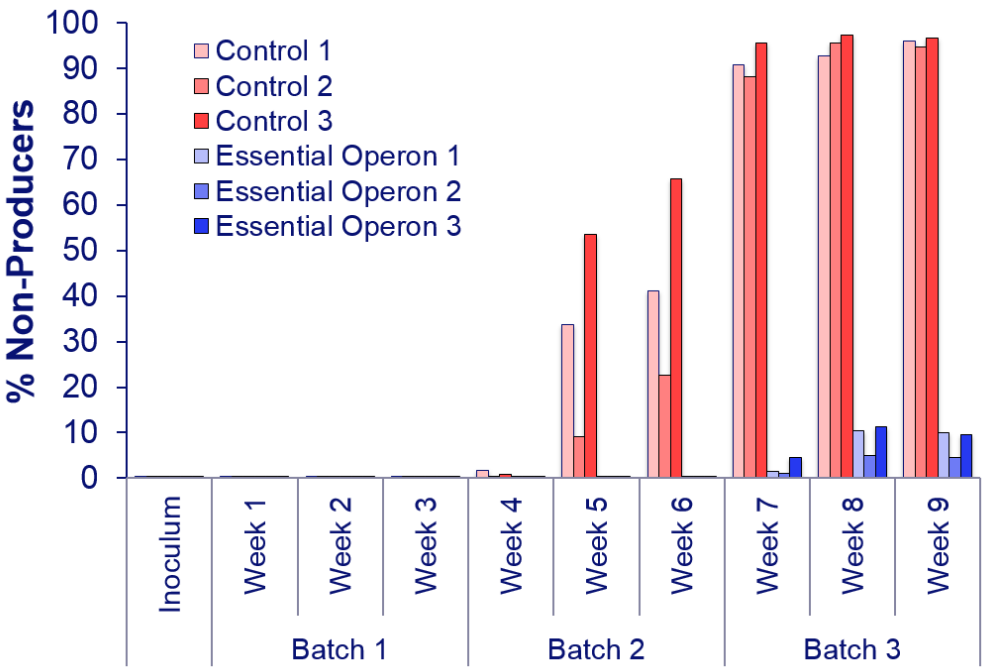


Figure A-18. The number of revertants (ethanol non-producers) present at various points in the cultivations shown in the preceding chart (Figure A-17).

It may also be possible to create strains that have higher genetic stability by knocking out or downregulating the transposases that are the most problematic. Elimination of error-prone DNA and RNA polymerases or other mutation-generating systems is also a possibility. Finally, it also may be possible to add more robust proofreading functions and DNA repair enzymes to AB1.

Strain development summary

To summarize, Algenol's extensive experience in cyanobacterial strain development has led to better and better strains, not only from the perspective of ethanol productivity, but also with respect to traits that will enable commercial production, such as enhanced genetic stability and contamination control. The chart below shows the ethanol productivity under standardized laboratory conditions for various strains produced over the course of several years. These productivity increases have largely come about *via* improvements in the ethanol cassette, which have led to the diversion of a very high proportion of the fixed carbon into ethanol. Algenol molecular biologists are now focused on further enhancing the production strain to improve overall photosynthetic carbon fixation rates, where we believe significant additional increases in productivity can be realized.

Improvements in ethanol productivity in laboratory cultures over the course of two years are shown in Figure A-19. These increases were primarily attributable to improvements to the ethanol cassette and are accompanied by a corresponding increase in branching ratio (up to ~80% at the end of 2013).

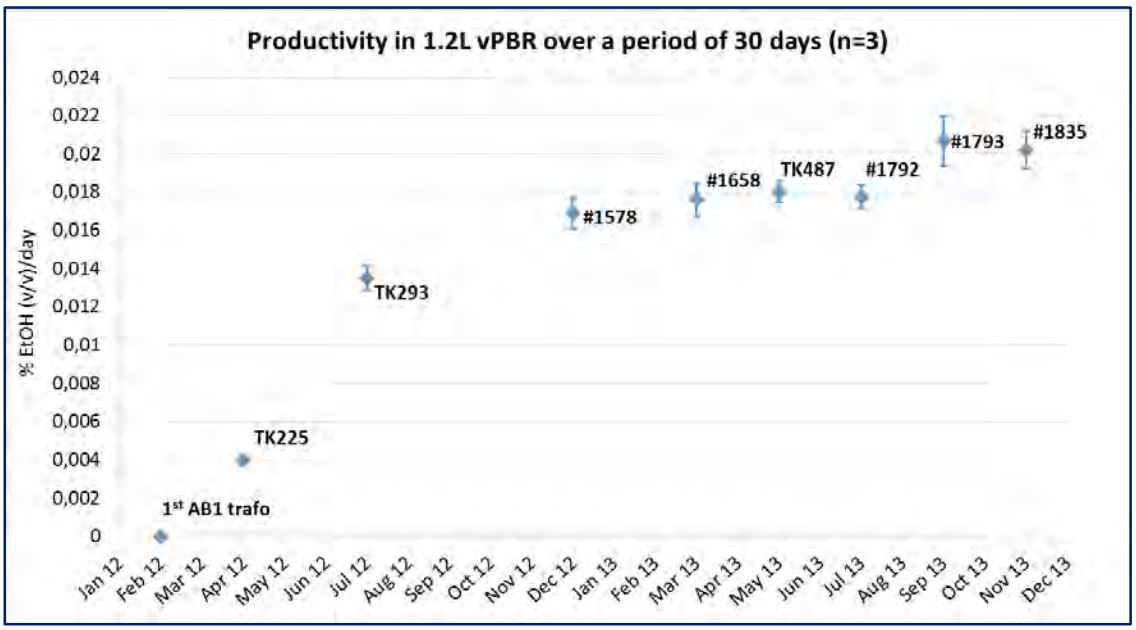


Figure A-19. Ethanol productivity over time for various AB1 derivatives transformed with the plasmids indicated in the figure. Strain designations for these plasmids are: AB0015 (AB1:TK293), AB0004 (AB1:#1578), AB0005 (AB1:#1658), AB0649 (AB1:TK487), AB0192 (AB1:#1792), AB0193 (AB1:#1793) and AB0214 (AB1:#1835).

Strain Characterization team

The Strain Characterization project team is responsible for performing more detailed characterization studies with promising strains in order to identify targets for additional genetic improvement of the strains and to help inform the development of advantageous culture management strategies. In addition, this project team includes a Photobiology sub-team focused on understanding the capacity, dynamics, and regulation of photosynthetic carbon fixation in ethanologenic strains of cyanobacteria, with the ultimate goal of providing information and data necessary to overcome limitations to the overall photosynthetic carbon fixation process.

One important activity for this team is a detailed characterization and comparison of the prioritized strains that have been created by the Strain Development team using standard culture conditions that mimic those of commercial production facilities. To enable this, a laboratory vertical photobioreactor (LvPBR) system was developed that mimics the light field and aeration characteristics of VIPER PBRs, but only contains a volume of ~1 L (Figure A-20). Standard conditions for illumination, media composition, CO_2 supply rates, and temperature are utilized which lead to ethanol and biomass volumetric production rates similar to those of outdoor VIPER PBRs. These standardized conditions also enable direct comparisons between results obtained in the Fort Myers and Berlin laboratories.

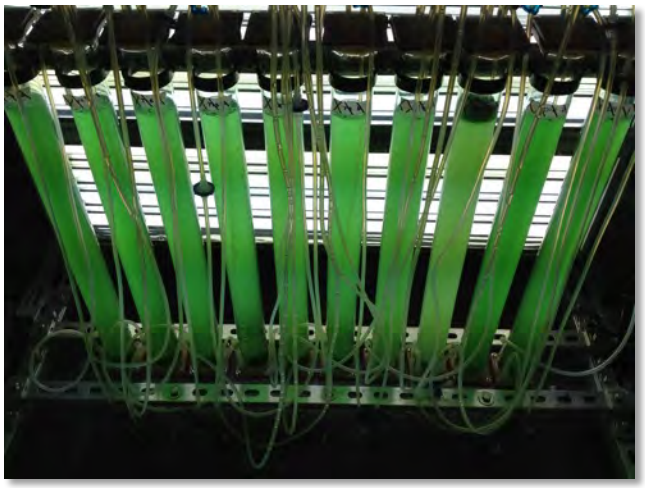


Figure A-20. Photograph of Algenol's LvPBR lab scale photobioreactor system.

Availability of the LvPBRs, along with a smaller volume version referred to as mini-LvPBRs, has enabled detailed testing of ethanol and biomass productivity of strains grown under different culture conditions, including gas supply rates (both in terms of overall bubbling rate as well as the percentage of CO_2 and air in the gas stream), nutrient supply, ethanol induction conditions, illumination, etc.) in a well-controlled and reproducible manner. The results of these studies are then supplied to the Field Cultivation team in order to implement the preferred conditions in outdoor cultures. The LvPBR platform has been shown to be a good predictor of strain and cultivation strategy performance at large-scale, outdoor VIPER PBR systems. See Section A.12 for more information on ethanol productivity and culture performance in saline water across various scales at Algenol's R&D facility (sub-task A.2.2), including studies to optimize nutrient supply at scale (sub-task A.2.3).

The Strain Characterization team utilizes state-of-the-art technologies to better understand the internal workings of the cells during the scale-up and ethanol production phases of the growth cycle. These technologies span physiological, biochemical, and genetic analyses, and are designed to shed light on the changes that occur within an ethanologenic host strain throughout the duration of the culture in order to establish methods to increase the extent and duration of ethanol production. The key to this approach is to understand the limiting factors for ethanol biosynthesis and overall growth. The following types of methodologies are utilized to identify these limiting factors:

Enzyme assays: the activities of a number of enzymes involved in carbon fixation, central carbon metabolism, and ethanol production have been monitored using *in vitro* assays in order to determine what the enzymatic capacity for a particular biochemical step is. In addition, quantification of enzyme and protein levels is often determined by the use of gel electrophoresis and immunoblots.

Isotope labeling studies and pathway analyses: The *in vitro* assays described in the previous section provide a good measure of the potential enzyme activities present within a cell, but are not necessarily accurate indications of the actual rate of enzyme catalysis within the cell because the rate is influenced by substrate and cofactor concentrations, feedback inhibition, and post-translational modifications that aren't necessarily manifest *in vitro*. Thus, Algenol scientists have made considerable efforts to develop methods to study cellular metabolism *in vivo.* These efforts include the use of C-13 labeling to follow the path of fixed carbon through the various metabolic pathways of the cell. Algenol has worked with external consultants who are experts in flux analysis to attempt to develop metabolic models for wild-type and ethanologenic

cyanobacteria and to determine the impacts of culture age and environmental conditions on metabolic flux. We were not successful in developing full metabolic models because of not having all the inputs and outputs necessary to close the model; the labeling patterns suggested complex networks of intermingled metabolism.

Metabolomics: To gain insights into the pool sizes of key metabolites along the pathway to ethanol, Algenol has worked with outside service providers on "untargeted" metabolomics projects to establish changes in various metabolite levels in cells in different stages of the growth cycle or under different growth conditions. We have also established procedures in house for "targeted" metabolomics, focused on those biochemical intermediates of the highest importance to Algenol's process. These analyses take advantage of the sophisticated analytical chemistry lab's capabilities, as described in a later section.

Genomics, Transcriptomics and Proteomics: The availability of the genome sequence of AB1 and other potential host organisms has allowed Algenol scientists to design and fabricate DNA microarrays for use in transcriptional profiling experiments ("transcriptomics") that provide the ability to examine expression levels of every single gene in the organism at any given time and under any cultivation condition, which is extremely useful for understanding cellular metabolism and informing the development of strain improvement strategies. The utility of the genome sequence and microarrays was further embellished by leveraging modern "next generation" RNA sequencing (RNA-Seq) platforms to further define gene structures and transcriptional start and stop sites. Algenol has also worked with service providers in "proteomics" projects to examine global changes in protein levels under different culture conditions. Although a detailed review of the results are not within the scope of this report, it is worth noting that these "omics" projects led to the identification of numerous potential gene targets to exploit for improved ethanol production.

Bioinformatics

The Bioinformatics department at Algenol has developed various databases and tools to accelerate the strain development and characterization process; these are found in the "ActHub" portion of Algenol's intranet. Central to ActHub is the Algenol Bioinformatics Portal, which contains the sequences of 16 proprietary cyanobacterial genomes that have been completed by Algenol as part of our quest for the best cyanobacterial host strain possible. The Bioinformatics Portal is essential because it contains genome information not available anywhere else in the world, but more than that, care has been taken to ensure that the annotation of the genomes is of the highest quality and is updated regularly. Useful views of these data include graphical overviews of the genes in genomic context, as well as various lists of genes grouped and sorted in a variety of useful ways. Links to important public databases of cyanobacterial genomes (Cyanobase), metabolism (KEGG), and protein families (InterPro) ensure that a wealth of information about each of the genes is easily accessible through web links. Custom developed tools for visualizing RNA-Seq, microarray, proteomics and metabolomics data in the context of the relevant biological pathways aid scientists in the interpretation of these complex data sets. Finally, the portal is completely searchable to ensure that this wealth of important information is available at the scientists' fingertips.

Other databases on the ActHub serve a vital role in organizing important information and making it available within and across scientific disciplines. The Strain Database tracks the many strains which were isolated from nature or acquired from culture collections, culminating in Algenol's well-studied production strain, AB1. The Constructs Database tracks the molecular biology constructs created by the Strain Development team to improve productivity and other relevant traits. Finally, the Technical Reports Database collects all of the technical reports produced by Algenol (over 600 to date) into one easily accessible, searchable location.

Analytical chemistry

Over the past several years, Algenol has established a world class Analytical Chemistry laboratory that is able to analyze many different aspects of both upstream (algal cultivation) and downstream (harvesting and biofuel recovery and purification) operations. The team performs routine analyses and specialized testing to support research, development and commercialization activities. The team has specialists in analytical biochemistry and chemistry who work in very well-equipped laboratories. Methods are developed and validated to enable written Standard Operating Procedures that include system suitability and check standards to ensure that accurate and precise data are acquired, while also providing information for investigating unexpected results. This approach builds quality into all the assays conducted in the laboratories. Highly specialized and short-term work is strategically outsourced to expert labs to assure expeditious and quality data.

The analytical chemists at Algenol have developed several routine assays for various nutrients that are important for growth and ethanol production (e.g., phosphate, sulfate, nitrate, nitrite, ammonia, and urea). The team is continually re-evaluating and improving these assays by either changing the format or technique to better meet the needs of the scientists and to improve throughput, accuracy, and precision. Nutrient analyses have evolved from flow injector analyzers to robotic 96-well plate formats to ion chromatographic techniques. Ethanol measurements are performed on state-of-the-art gas chromatography-headspace systems that have the necessary precision and accuracy but can still be run in an automated, high throughput manner. Gas chromatography has been used to measure other volatiles that are important in the cells' metabolism. Semi-volatiles and non-volatiles are analyzed by liquid chromatography.

The liquid chromatography platform is varied and can support analyses of diverse types of molecules. Ion chromatography, reverse phase, hydrophobic interaction, ion pairing, etc. along with specialized detectors (multichannel UV, charged aerosol, electrochemical, photodiode array, mass selective) has enabled the team to develop methods for numerous compounds of interest. The acquisition of a Triple Quadrupole Time-of-Flight mass spectrometer expanded the analytical lab's capabilities to do targeted metabolomics and carbon-13 flux analyses in house. We have a developed several platforms to measure pool sizes and isotopomer patterns of key central metabolites and surrogate molecules. These analyses assist the Strain Development team to choose and evaluate targets for improving production strains.

The department also has strong capabilities in spectrometry to assist the Engineering and Operations teams for material and product analyses, including chemical profiling of product streams from hydrothermal liquefaction. These tools include mass spectrometry, FTIR, and UV-VIS-IR, and fluorescence spectroscopy. The following is a partial list of the physical assets on location at the Fort Myers Analytical Chemistry Laboratory:

- 3 Agilent GC-FID Systems, 1 GC-MS System
  - Headspace, liquid, and gas stream sampling
  - Measurement and identification of volatiles
- Shimadzu TOC-V Analyzer
  - Measures inorganic and organic carbon as well as nitrogen from liquid samples
- Waters Acquity UPLC with UV/Vis Detector
  - Reversed phase, size exclusion, ion exchange, hydrophilic interaction
  - Analysis of peptides, proteins, amino acids, small molecules
- Sciex 5600 Q-ToF with Waters Acquity UPLC
  - High resolution quadrupole time-of-flight mass spectrometer

  - Accurate mass instrument capable of quantitation and MS/MS identification, ESI, APCI
  - Proteomics, metabolomics, lipidomics, ^{13}C labeling, protein identification, structural elucidation
- Thermo iCap 7600 ICP-OES
  - Liquid autosampler, microwave digester
  - Trace metal analysis and quantitation
- 3 FIA Lab Flow Injection Analyzers
  - Flow injection chemistries for urea, ammonia, and phosphate analysis
- Thermo FLASH 2000 Elemental Analyzer
  - CHNS/O analysis of solid samples for compositional analysis and total protein determination
- Thermo Dionex ICS-5000 and ICS-2100
  - Ion chromatography for determination of anions and cations in solution, nitrate, nitrite, organic acids, sugars
- Mettler-Toledo V20 Karl Fischer Titrator
  - Water content in organic solutions
- Mettler-Toledo Excellence Titrator T7
  - Automated titrator with autosampler
  - Acid/base content, chloride determination
- Beckman-Coulter Biomek FXp
  - Robotic pipettor for 96-well plates, multiple size tubes, includes shaker and Peltier chiller
  - Sample preparation, ELISAs, container reformatting
- Netzsch TG209 F1 Libra Thermogravimetric Analyzer
  - Automated TGA with evolved gas analysis capability
  - Useful for material identification, sample ashing
- Mettler-Toledo HS153 Moisture Analyzer
  - Total dissolved solids, dry weight, moisture content
- Thermo Nicolet 6700 FTIR with Attenuated Total Reflectance apparatus
- Biospec Mini-bead beater for cell lysis
- Cary 300 12 cuvette chamber UV-Vis Spectrophotometer (enzyme kinetics)
- Molecular Devices SpectraMax 190E, M3, and M2e microplate spectrophotometers
- Perkin Elmer 650 UV-Vis with Internal Sphere (reflectance measurements)
- Brookfield DV2T Viscometer
- Dionex ASE 350 automated solvent extractor for materials characterization
- BioMek FXP Robotics for automated sample preparation
- metabolite platform)
- Biospec Mini-bead beater for cell lysis

Algenol has developed a LIMS using Microsoft SharePoint for sample tracking in the Analytical Chemistry department. The system consists of an experimental summary form which initializes the workflow. The experimental summary provides the group with the anticipated assays, number of samples, and duration of the experiment and launches the creation of a data repository with access to the principal investigator, experimental leads, and any other personnel deemed necessary. Several forms for the different analytical test methods (ATM's) are available for submitters to fill out when dropping off samples for analysis. Each of these forms tells the analyst the number of samples, the project, and the type of assay. These forms have the ability to track samples as being submitted, in-process, or complete. Not only does this help analysts organize and track the status of thousands of samples for multiple assays, but it informs the submitters when their data are available and ready to view. Quality control checks, standard values, and trend analysis are collected and help monitor data quality and assay wellness and can be viewed at a glance using control charts updated in real time. In the background, the system is also tabulating the number of samples for a given assay, project, submitter, and timeframe which is sortable and filterable. This feature allows Algenol to report exact analytical numbers and predict resource allocation, bottlenecks, and costs.

Subtask A.3 Flexible film photobioreactor development

Completed July 2011

Horizontal bioreactor development

The horizontal systems were developed to provide a means of growing algae on a large scale in closed PBRs. They were designed to be as inexpensive and energy efficient as possible, and because they were closed, provided a means of collecting ethanol vapor as condensate on their walls. The basic commercial design was a 50 ft tube of 0.008" multilayered polyethylene film inflated with about 0.5" WC and filled with 4500 liters to hold a 200 mm deep culture. The culture was mixed with a magnetically coupled hydraulically driven foil array (Figure A-21). The array was eventually manufactured as a single blow molded part which could float on the surface of the culture.

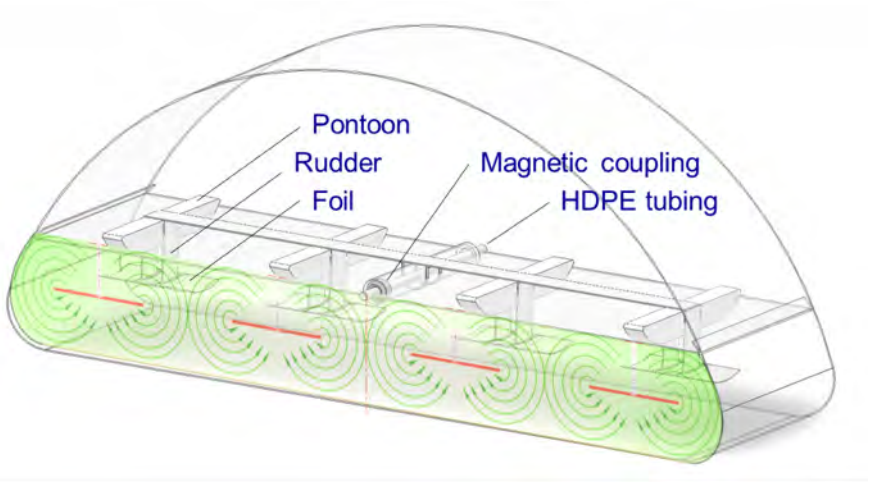


Figure A-21. Magnetically coupled mixing system in cross-section of horizontal bioreactor. Foil mixing generates a set of persistent stationary vortices for maximum vertical flux.

Development proceeded on many fronts. The core research was devoted to understanding if the effect of the vortical mixing could increase productivity. Experiments bore out that the 30 second mixing timescale did not improve productivity, but was necessary to prevent settling of the cells. Later experiments, published in our patent for the system, (Miller, III (U.S. Publ.

2012/0220027)), showed that productivity was more than paddlewheel systems when providing the same energy for agitation, but the major improvement was lower capital costs. Horizontal systems would be a tiny fraction of an encapsulated paddlewheel design, with the same or equivalent bioreactor area on the scale of hectares.

A parallel course of development investigated the commercial viability of the PBR and mixing system. A hydraulic system was designed that incorporated a pair of Neodymium rare earth magnets (cylinder and doughnut) that were encapsulated in ultra-high molecular weight (UHMW) plastic designed to reduce wear and prevent corrosion. Much effort went into making sure the system could work on a commercial scale, with a single hydraulic drive moving arrays of mixers in series (Figure A-22). One system composed of 40 PBRs with hand built mixers was installed and tested with water for more than a year. This showed the condensation collection effectiveness and the robustness of the final design while also highlighting some of the design challenges. The most prominent of these was leaking PBRs. The tolerances of the internals of the hydraulic system generated sufficient leakage that losses due to the hydraulic fluid movement were deemed untenable for our energy consumption targets. A pneumatic design solved the energy problem. It also required less expensive magnets. This design was deployed with the commercially produced blow molded “boats” carrying the foils in 4 50 foot PBRs in late 2011. It was run for a month. Running continuously at 0.5 m/s with a 2.7 psi supply pressure and 6 SLPM/reactor, it consumed less than 4.3 W/23.3 m^2 PBR (0.18 W/m^2) assuming a 50% efficient compressor.

Experiments later indicated that high levels of oxygen super-saturation existed in these cultures, presumably restricting production. This spurred development of fast moving skimboard systems (foil array removed) which produced greater agitation of shallow cultures (50 mm deep) for de-gassing. This resulted in a design that was much more effective than conventional technologies in terms of aeration/m^2 for the energy expended, but unfortunately was not patentable. The results of these tests did not show a major increase in ethanol production over the course of a batch compared to vertical (light dilution) experiments, so the horizontal PBR projects were halted. Algenol’s direction then turned toward vertical bioreactors that were mixed with bubbling from air diffusers.

Figure A-22. Pneumatically driven horizontal mixing system with blow molded mixing elements.

Flexible film vertical bioreactors

The development of vertical photobioreactors was driven by the need to improve light utilization and oxygen and temperature management in outdoor cultures. The initial studies were focused on understanding the relationship between PBR configuration and operation and ethanol production. Two outdoor deployments of vertical bioreactor systems had demonstrated measurable increases in ethanol productivity in rudimentary vertical systems. These early deployed PBR versions were not optimized, but provided quantitative results on how light utilization improves biological performance. R&D teams of engineers and biologists conceptualized and evaluated more than 10 versions of vertically oriented PBR designs, but deployed only 3 prototypes for outdoor testing. The increase in productivity over traditional horizontal systems was demonstrated both for ethanol and biomass yields per PBR footprint. Vertical PBRs have a temperature mitigation advantage over horizontal PBRs likely due to indirect light exposure and larger surface area to volume ratio. Higher oxygen transfer rates and improved oxygen management were also achieved in these systems. As an example, the volumetric gas transfer rate in the vertical system was found to be 6 times higher when compared to horizontal systems. These tests were conducted by bubbling oxygen in water for a range of designs.

First generation vertical photobioreactors (VIPER 1)

The use of flexible film for vertical bioreactor manufacturing necessitated some form of structural support. The first set of PBRs were placed on the ground within a metal frame and supported on the sides with either a wire mesh or polycarbonate sheet. This method was considered to be extremely costly and inefficient, so the company sought ways to reduce the cost associated with supporting the PBRs. The first generation vertical PBR (VIPER 1) was designed to be partially self-supporting, requiring no frames to maintain the required shape. The distance between seams provided good control for panel thickness and could be used to optimize optical depth, mixing and gas exchange.

Hybrid vertical flexible film PBRs

One design approach for bioreactor design was to incorporate the advantages of vertical PBR concepts into Algenol's original horizontal PBR configuration. The aim was to increase surface area to volume ratio, achieve light dilution, and increase light utilization, while eliminating the need for elaborate PBR support and attempting to maintain the passive condensate collection process for ethanol recovery. There were two hybrid designs. The first was an array of first 6 (and later 13) vertical panels enclosed within a traditional horizontal flexible film PBR. The equally spaced inflated plastic panels were seamed at the base of the horizontal PBR enclosure (Figure A-23). The second hybrid design was a light pipe PBR of similar construction to the horizontal PBRs. A series of hollow plastic tubes were incorporated into the PBR to channel light into the medium. The tubes acted as light guides collecting irradiance above the culture surface and distributing it into the culture volume along the tube-culture interface, decreasing the effective culture depth and increasing the illuminated surface area of the system (Figure A-24). In both cases, air was bubbled into both designs to mix the culture and promote efficient gas exchange. Algenol's hybrid photobioreactors were intended to improve the existing horizontal PBR design by allowing improved light utilization.

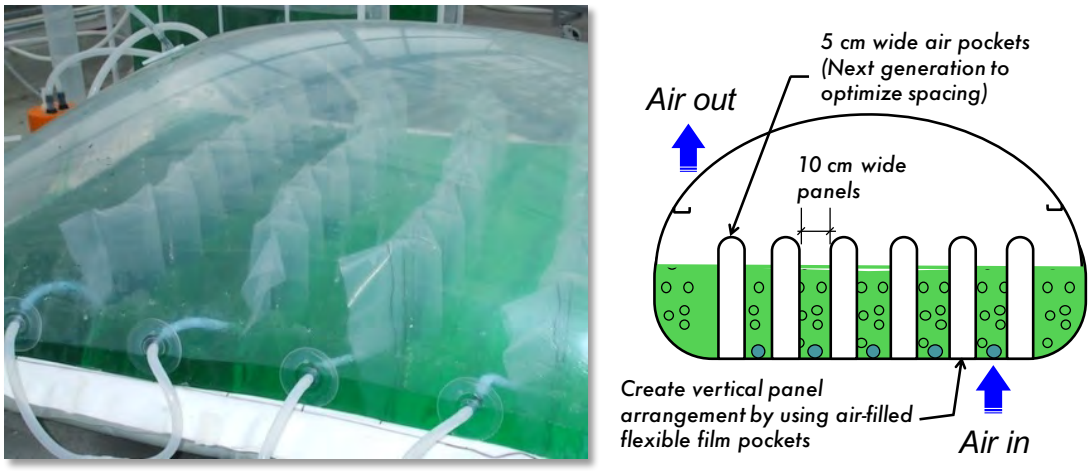


Figure A-23. Left – photograph of hybrid PBR with inflated panel separators within a horizontal PBR enclosure. Right – Schematic of hybrid PBR system.

Figure A-24. Light-guide PBR deployed outdoor at Algenol.

Rigid vertical photobioreactors

While vertical PBRs demonstrated quantifiable increases in biomass and ethanol production over their horizontal predecessors (see section A.12), flexible film PBRs require installation of a support structure, which could be cost-prohibitive. Therefore, there was significant interest in eliminating the need for external structural support. This led to research into the use of clear rigid materials for PBR construction. In reality, the approach was to shift the cost from the external support to the bioreactor material itself and explore other benefits to make the entire system more cost-effective. For example, extending PBR lifetime through the use of rigid panels is one such potential improvement. PBR setup and operation could also be optimized or changed to improve costs.

Three materials were evaluated for rigid panel construction: polycarbonate (PC), PET and acrylic. For R&D purposes, acrylic was chosen because it was readily available in the desired dimensions. However, its potential for large-scale deployment was highly doubtful given the cost and material brittleness. PET and PC are cheaper, more impact resistant, but would require custom construction. Rigid panels were made from 4-ft squared Acrylite Alltop clear extruded acrylic panels with channel dimensions of 16 mm depth × 64 mm width, and thickness ranging

from 0.15-0.20 mm (60-80 mils). Due to this acrylic's high cost, other materials such as PC and PET were being investigated for future rigid PBR builds. Acrylic square tubes and a PC U-channels were used on the panel's bottom and sides, respectively. The PBR cap was enclosed with 2 channels cut from a full panel and placed horizontally. Three polycarbonate quick connectors were attached to this top piece for sampling and sensors. Four 3/8" polyethylene barbs connected adjacent PBRs at the top for headspace and liquid overflow (Figure A-25).

Figure A-25. PBR cap and air-out header with stabilizing structure.

Sixteen panels were deployed in a "W" shape with alternating 90˚ faces, and spaces between panels running east-west. Spacing was set at 2 feet, face to face. Four simple dye panels were incorporated into the block on the south facing side for representative shading (Figure A-26). The air-out headers were connected to containers for foam collection. Two drain headers were run underneath PBR nodes and allowed for selective communication of up to 8 PBRs by the use of inline valves. Tubing connects paired panels to drain headers. Ball valves were also plumbed at the end of each drain header.

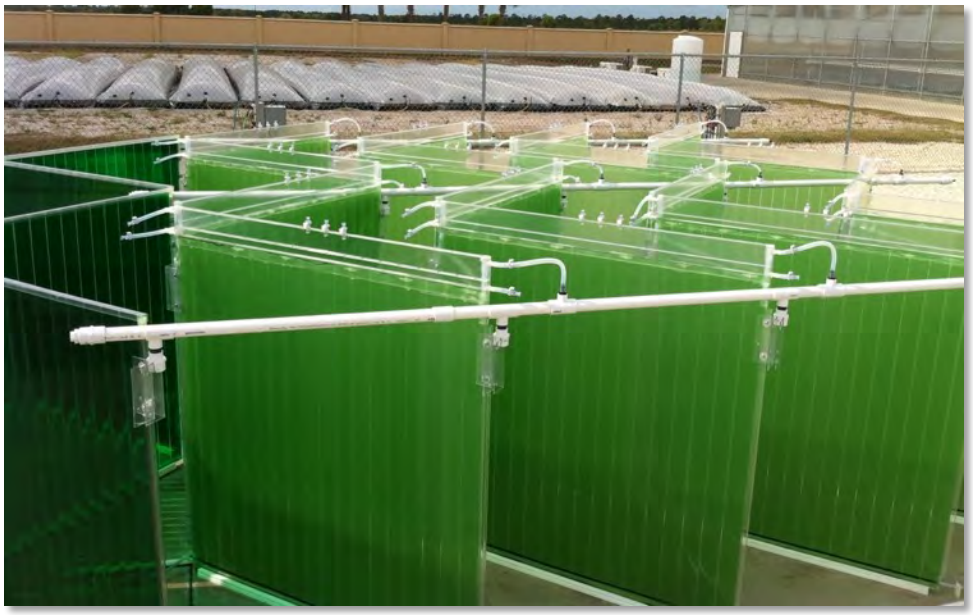


Figure A-26. A west facing photo of dye panels and cultures on day 2

VIPER 2 vertical PBR system development

VIPER 2.1 – The drive behind the second generation of vertical photobioreactors was focused on developing industrial scale systems for commercial deployment. Concurrent with VIPER 2 design was the development of the commercial manufacturing method. R&D efforts were specifically targeted at improving the economics of the ethanol production process compared to first generation VIPERs. The goal was to advance the cultivation field with PBR design improvements through reduction of total gas flow, piping in the fluid and gas distribution networks, number of ports and connections, and improvements in volumetric and areal productivity. Efforts were also focused on improving PBR deployment, media delivery, PBR mixing, culture management, CIP, and reducing the amount of plastic used per PBR on an area basis. Between June 2013 and February 2014, over 50 different prototype vPBRs were developed and built. The design team operated 11 different prototype designs outdoors in 5 independent experiments. Some critical parameters evaluated included PBR thickness, spacing, PBR weld pattern, height, width, and CIP.

VIPER 3 vertical PBR airlift systems development

Second generation vertical PBRs were operated as independent panels in a large array (>100). This required delivery of nutrients to individual PBRs, which was often difficult to achieve with high precision. Further, the isolation of these PBRs came at a relatively high cost. Therefore, the company sought to reduce PBR isolation in order to reduce cost and improve culture management operation. To achieve this, a series of PBRs were connected together so that the culture could be circulated within a larger unit. An airlift pump was connected to the main fluid headers and was used as the driving force to move liquid through the headers. The introduction of air in the airlift column provides the driving force for moving liquid around in the system. Relatively high recirculation rates are achievable by careful selection of the airlift column diameter, the airflow rate, and the system pressure drop.

Bioreactor manufacturing equipment development

VIPER manufacturing – A critical element of successful commercialization of PBR cultivation systems is the capability to manufacture large quantities of PBRs with high quality at low cost.

As the PBR design moved to the vertical orientation, the manufacturing technologies required for production of the PBRs also changed. Initially, a medical device company was engaged to support the PBR demand through the deployment of the 4000-Block. This vendor produced PBRs with marginal quality at high cost, so Algenol decided to develop the manufacturing capability in-house.

Sub-components, tube kits and header manufacturing – Manufacturing systems were also developed for diffuser assemblies. The diffusers have been designed with specific material and hole frequency and dimension requirements. Custom equipment was designed to allow punching and cutting to length of the diffuser tubing. These components are then transferred to the diffuser assembly area in the plant, where the remaining diffuser components are added to produce a component ready for installation into the PBR structurally welded component. The gas delivery end of the diffusers is welded into the PBR side seam, providing the Air In connection point. Diffuser design modifications are ongoing and include development of low cost options, allowing automated production and minimal assembly steps.

PBR film performance improvement

At the outset of this project, the plastic film used to construct the horizontal bioreactors was a material that had been specially formulated to withstand years of exposure to solar UV while maintaining its clarity, and also contained some proprietary additives to reduce condensation. Chemically the film was primarily polyethylene and included small amounts of UV blocking additives that reduced the transmission of wavelengths below 390 nm to very low levels.

The transition of the PBR design to a vertical hanging orientation added many new design requirements to the film structure, especially related to dimensional stability: the film needed substantial resistance to creep, in addition to the UV resistant properties. Concurrently with the PBR design orientation change, a high quality and high speed manufacturing methodology was developed for welding the opposing faces of the PBR together while forming the edge seams. This methodology uses heat, and therefore, sealability of the film became an added design requirement of the film structure.

Algenol continues to add performance requirements to the film as necessary. The current specification includes:

1. Measures of film mechanical properties:
   - Yield strength
   - Rupture strength
   - Stiffness
   - Toughness
2. Measure of sealing characteristics
   - Seal strength
   - Burst strength (water pressure head above full fill)
3. Optical
   - Haze
   - Opacity
4. Economics
   - Cost per PBR
   - Vendor performance (i.e., customer service, quality, lead times, etc.)

Due to the challenging application of 6 years expected life outdoors, Algenol enlisted the services of multiple resources, including Polymer Suppliers, Additive Suppliers, and Blown Film Manufacturing Companies to develop a long life, tough, weldable, clear, biocompatible, low

creep, and flexible film for PBR construction. The best candidates of these films have been subjected to the equivalent of 6 years of UV exposure in UV chambers and maintain sufficient performance to provide high confidence in their outdoor performance capability.

PBR system design improvement and cost reduction

Overview – In mid-2014, Algenol began making significant strides towards reducing the cost of the cultivation system in an effort to hit commercial cost targets (Table A-3). The first components targeted were the PBR and the associated tubing kits, which connect the PBR to the piping infrastructure to enable fluid transfer. A three-fold reduction in cost was realized with an approach that identified commercial vendors who could supply materials and services at cheaper prices (including volume-based reduction), and modification to manufacturing and design. In 2015, the same approach was applied to the support structure and piping to explore cost reductions for these components.

The cultivation system includes three functional groups; (1) the PBR (including the diffuser and tubing kits), (2) the piping infrastructure (including headers and manifolds that move liquids to and from the field), and (3) the support system that holds the PBR in its vertical orientation. The shift from VIPER 2 to VIPER 3 already led to a 50% cost reduction after volume-based costing was applied. The cost analyses were based on real systems deployed at Algenol IBR and PDU platform. The piping system comprises mostly HDPE pipes. The support structure is mostly a galvanized steel frame. The PBR is secured to the frame with a hollow electrical metal tube (EMT).

Table A-3. Cost reduction targets and analysis results based on work conducted as part of the R&D effort on PBR cost reduction. All costs are based on volume-based cost reduction and identification of commercial vendors to supply materials are scale with improved design.

Parameter	P10	P50	P90	1.8-acre IBR	Basis for Estimate
PBR Cost (10-ft basis)	$7	$9	$12	$47	Completed work in 2014
Support structure and piping	$3	$10	$25	$70	Preliminary analysis
PBR life (years)	12	8	6	4	New plastic

Target and Approach – The PBR Systems Team was formed in direct response to the output from the cost reduction effort of 2014. One of the primary purposes of the team was to work closely with manufacturing to ensure that Algenol's PBRs and cultivation system conform to design specifications and meet the techno-economic targets. In addition, the team worked with other stakeholders (IBR, Field Cultivation, and Inoculum System) to evaluate focused design innovations to improve PBR system functionality, enhance culture productivity, and reduce operational costs associated with algal cultivation (including CIP). The team also executed research and experiments to evaluate the feasibility of advanced PBR, diffuser, and PBR system designs that have been identified as significant in cost reduction or functional improvement.

The team developed and implemented a systematic design process to achieve cost reductions in the PBR system. The first step of the process was to document the functional and special design requirements for piping and support structure utilizing the *quality function deployment* (QFD) technique. In addition to the current state analysis, the QFD was important for identifying the specific parameters for redesign based on the cost reduction impact. A bill of quantities (including material and labor) was compiled for all components used in the piping and support structure, and commercial vendors were identified so that quotes for future demand could be obtained. The team then held brainstorming meetings and generated in excess of 50 different ideas to reduce cost in various areas of the system. These ideas were grouped into

major concepts. For the support structure, reduction in PBR weight, material, and construction labor were all significant cost drivers (Figure A-27).

Another concept explored was the complete removal of the support structure, such that only the cost of the piping is important. In this concept, the support function would be transferred entirely to the PBR by using a more rigid film and orient it in a stabilizing structural form. Since PBR life is a major driver in the overall economics of the cultivation system, the concept is particularly more appealing if increased PBR life can be achieved by spending a bit more on the film once structural costs are eliminated.

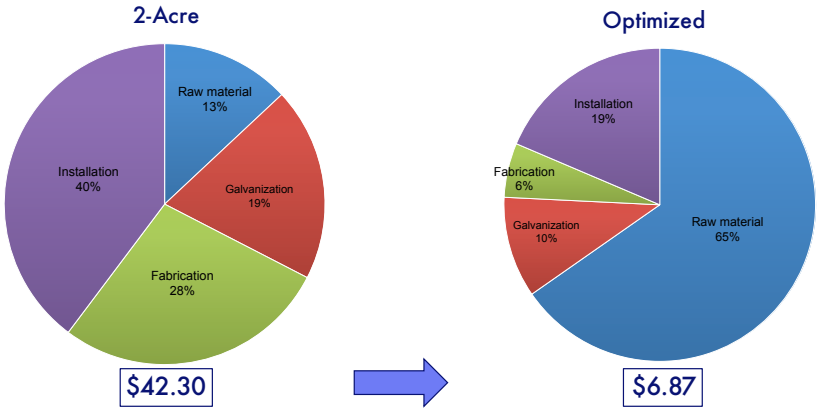


Figure A-27. Cost reduction potential for support structure. Optimized designed is based on the use of a 3-post system in which there is no welded components.

For the piping system, the goal was also to reduce the material and labor costs by utilizing thinner pipes with barbs already formed into them. Manufacturers with this capability have been contacted and arrangements are being made to prototype our proposed designs. The team is also working with the commercial team to explore block designs that reduce valves for overall cost savings.

The production cultivation system (assuming an airlift unit design) has a header piping system that includes four main headers: air-in, air-out, liquid-in, and liquid-out. They are connected to the PBRs via tubing kits. These connections must be completely hermetic and rugged, able to withstand the oscillation of the suspended PBR due to wind. The position, fluid flow, and pressure rating vary among these headers, conferring on each a specific set of requirements for servicing the PBRs. The other two headers, liquid-in and liquid-out, are located below the PBR. They must accommodate liquid flow rates sufficient to meet specific time requirements for operations, particularly fill and drain times.

Based on current designs (1.8-acre IBR), the PBR headers represent about 25% of the total system cost. To reach commercial targets, the PBR Systems team implemented a structured design approach. The process began with a clear definition of the design requirements followed by engineering specification development (Figure A-28).

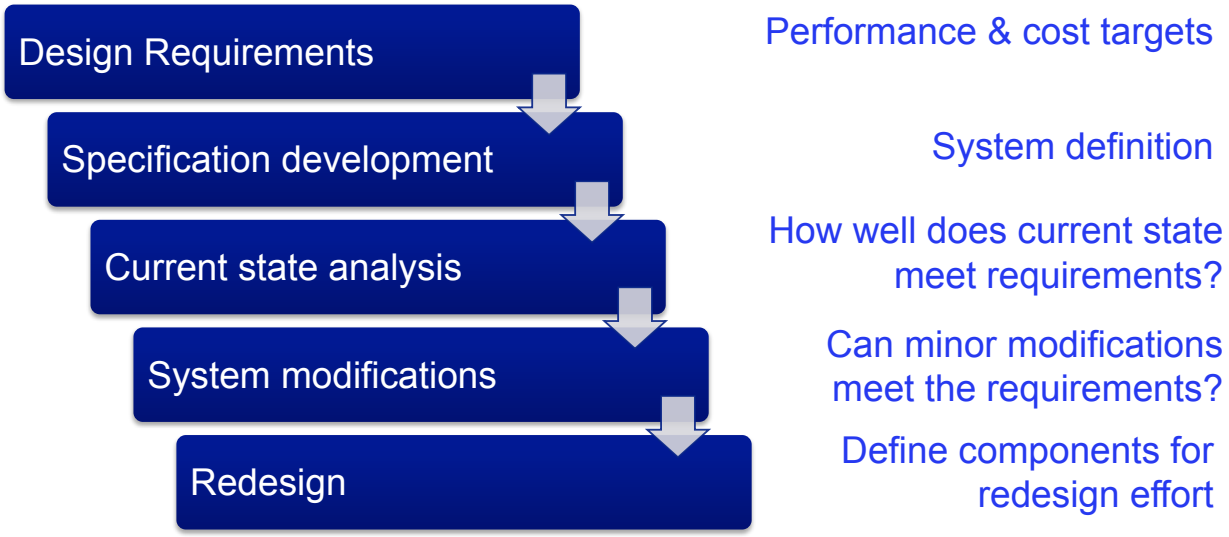


Figure A-28. Process summary for PBR Systems cost reduction program.

For existing systems, current state analysis gives a thorough cost breakdown and identifies opportunities for cost saving. As part of the exercise, vendors and volume-based cost reductions were first pursued. However, these did not yield significant cost reductions compared to the film and tubing kit project. Therefore, a search for material and parts substitution was important. The main goal was to obtain as many suitable products currently available on the market and assess their ability to meet the requirements of the piping system. Based on this effort the potential to reduce from $28.30 to $6.67 was determined (Figure A-29).

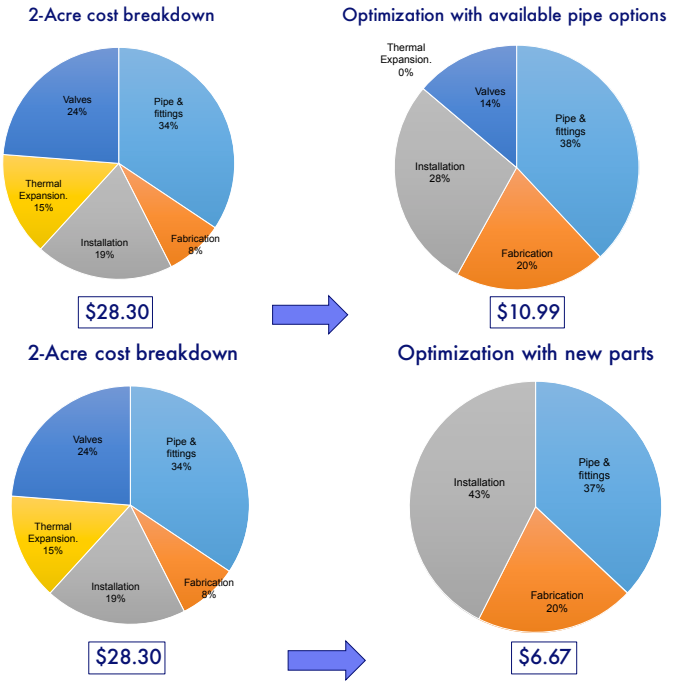


Figure A-29. Pipe cost reduction potential.

Diffuser cost reduction – Diffuser cost reduction targeted <$1.50 per 20-ft length of diffuser. This was achieved through design changes and replacement of the orifice. Figure A-30 below shows the cost reduction progression.

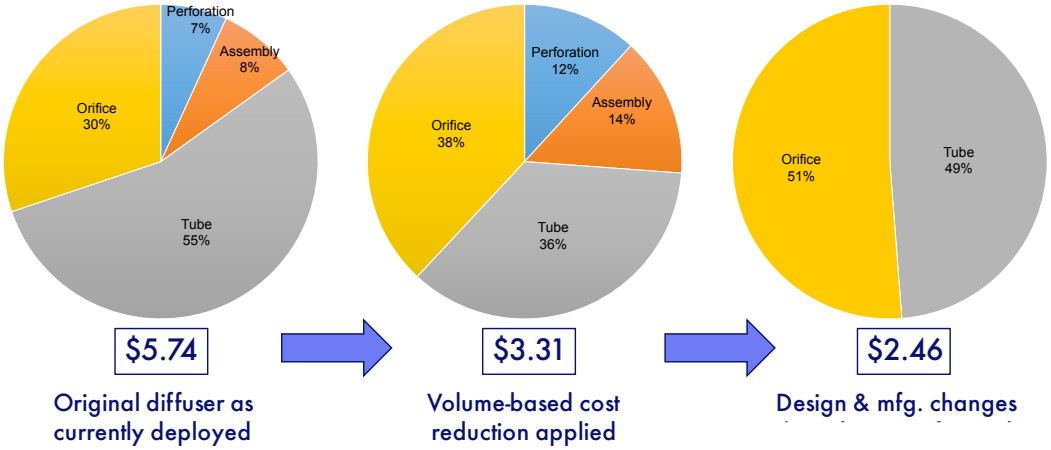


Figure A-30. Diffuser cost reduction.

Subtask A.4 Process engineering

Completed July 2011

Integrated Biorefinery process engineering

To properly describe the fully integrated ethanol production system, the Integrated Biorefinery (IBR) process flow diagram was divided into 11 areas as described in Table A-4. The relationship between these areas is depicted in Figure A-31. Over the course of this project, each of these areas was designed with the ultimate goal of integrated operation. All areas except 0500 were constructed and tested in IBR operation. The HTL unit for area 500 was constructed, but not testing with IBR feed. Feasibility tested for the HTL operation was carried out at Pacific Northwest National Lab (PNNL) with Algenol funding. Area 500 was not necessary in the original design described in the proposal. As can be seen from Figure A-31, the design and operation of the cultivation related elements are highly interdependent. The separation related elements are connected by mass flows only, and were evaluated as a series of batch operations.

Table A-4. Area definition reference table.

Area Number	Description
0100	Inoculum Field
0200	Production Field
0300	Dewatering
0400	Ethanol separation
0500	Hydrothermal Liquefaction (HTL)
0600	Nutrients
0700	Clean in Place (CIP)
0800	Gas Management
0900	Product Storage
1000	Wastewater Treatment
1100	Utilities

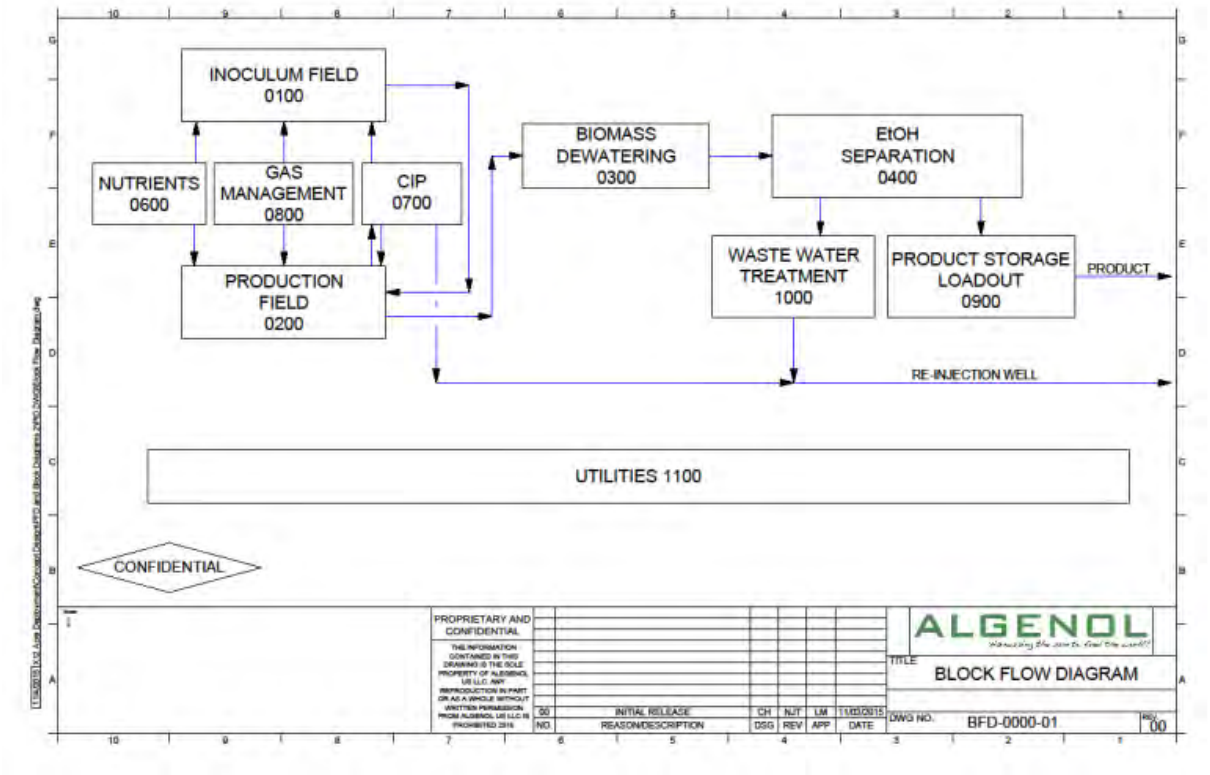


Figure A-31. Integrated process flow diagram.

Flexible-film photobioreactor cultivation systems

The design progression of the PBR is addressed in Section A.3. As was anticipated in the project proposal, the incorporation of those PBRs (whether horizontal or vertical) into the ethanol production system was a significant challenge. Design development required prototype development and repeated testing cycles to understand not only the component or system design performance, but also the integration effects (i.e., the effect on other design elements). The interface designs were also challenging, and required the development of a Basis of Design that linked the planned production rate of the cultivation (i.e., upstream) systems with the separation (i.e., downstream) systems.

The IBR cultivation field covered about 1.8-acres and was designed in late 2014. Smaller scale systems (400 Generation 1 VIPERS, and 4000 Generation 1 VIPERS) had provided considerable amount of design and operational data in preparation for the 1.8-acre cultivation system design and construction. The 1.8-acre cultivation system included newly designed PBRs, Vertical PBR Support Structure, Field Piping, a new Pavilion, and a Process Pad that contained the infrastructure required for areas 0100 (Inoculum), 0600 (Nutrients), 0700 (CIP), and 0800 (Gas Management). The design covered approximately 1.8-acres, including the new pavilion, and process pad. Figure A-32 shows the general arrangement.

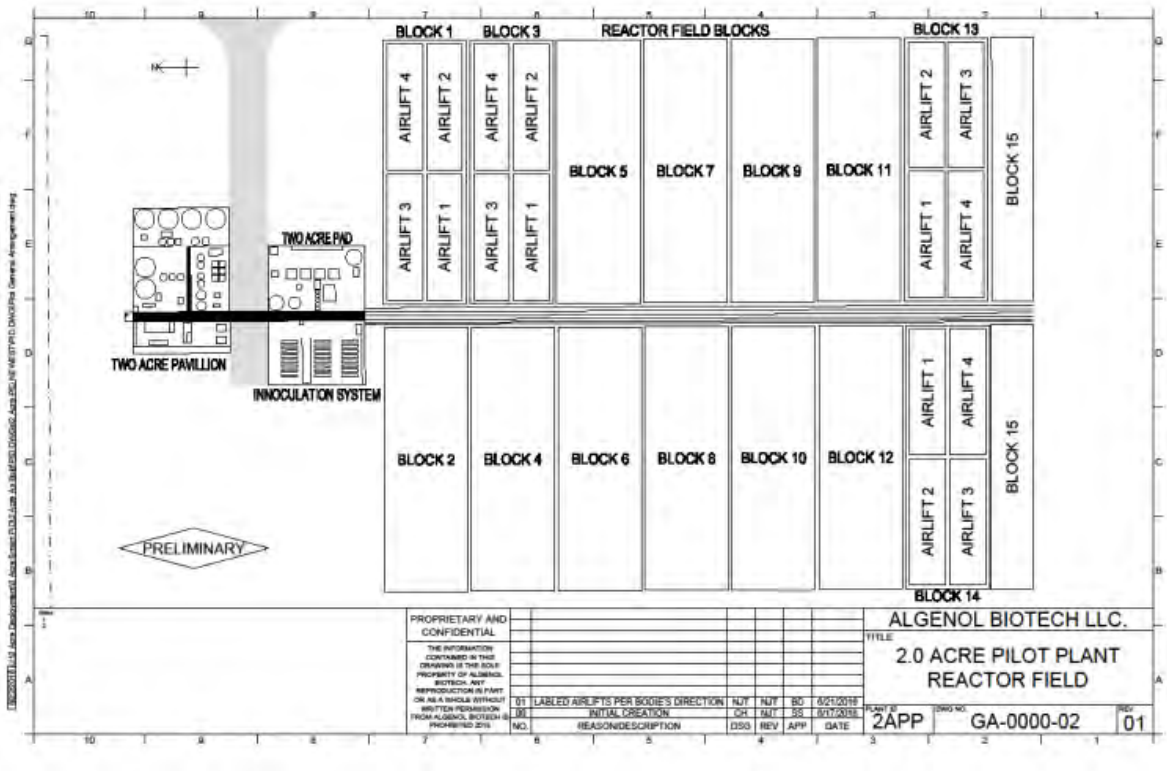


Figure A-32. 1.8-acre IBR general arrangement.

As can been seen from Figure A-32, there are two types of block identifications: “Block #” and “Airlift #”. The PBR designs and operational details differ between these designations, with PBRs in the “Block #” designated blocks, being VIPER 2; and “Airlift #” blocks containing VIPER 3.x. The harvest volume in either case was approximately. The original design intent was to alternatively cultivate each block for 27 days, then harvest, CIP and re-inoculate the block over 3 days, returning it to production. This philosophy would provide salt water, ethanol and biomass containing harvest to the biorefinery every 2 days. This volume of material harvested as discreet batches with the expected ethanol and biomass concentrations was used as the basis of design for sizing the ethanol refining equipment.

Biomass and ethanol separation at the Biorefinery

Figure A-33 shows the relationship of the Biorefinery to the Production Fields. As can be seen, the Downstream Pavilion, housing the biomass dewatering and ethanol separation equipment is reasonably close to the PBR Field Blocks (the Production Field), and the harvests were transferred from the PBR field to the downstream pavilion through underground piping.

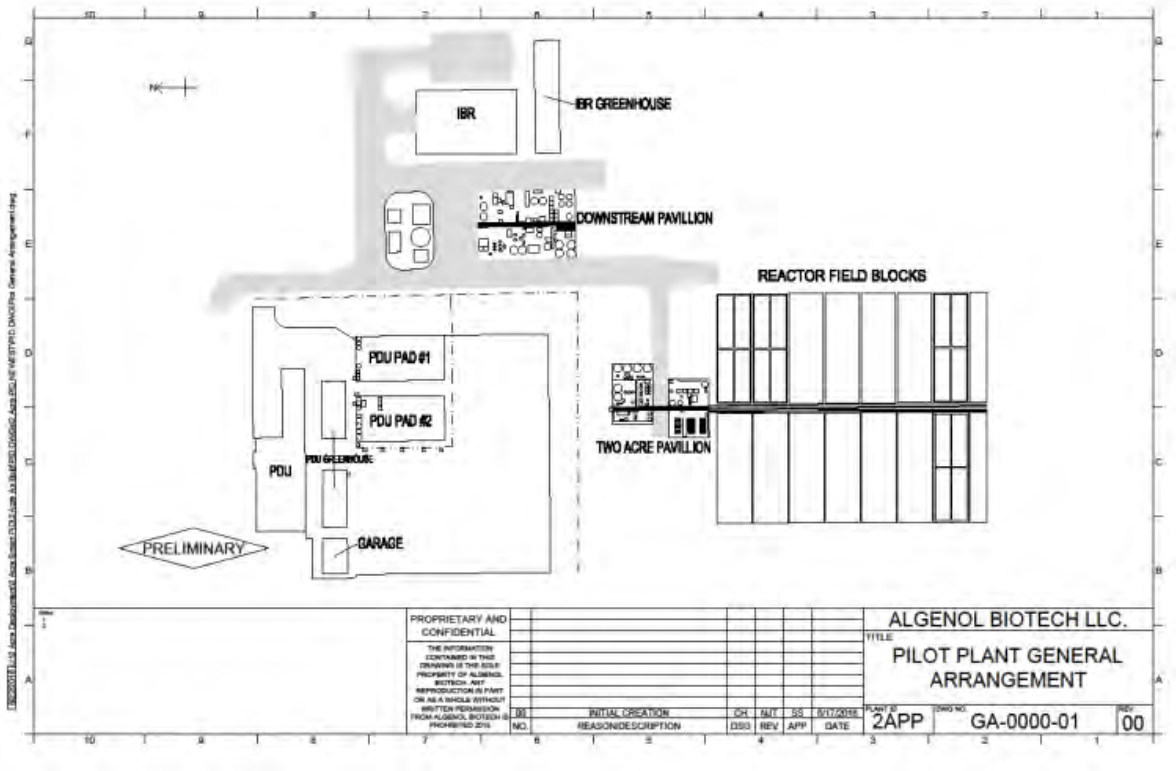


Figure A-33. Overall site general arrangement.

Figure A-34 shows the equipment arrangement within the Downstream Pavilion. The harvest enters and is staged for processing in the tanks designated “14”.

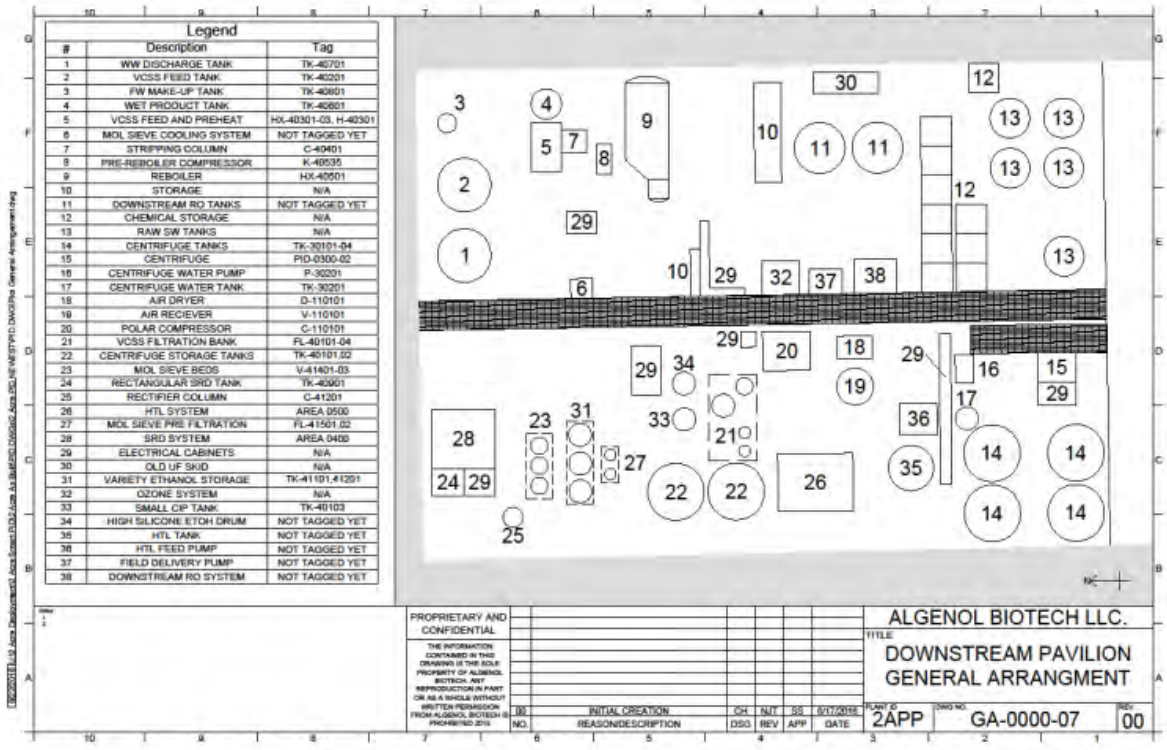


Figure A-34. Biomass and ethanol separation general arrangement.

The Piping and Instrumentation Diagram (PID) for the Harvest Storage Tank Farm is shown in Figure A-35. As can be seen from the PID, the harvest stream leaves tank farm and proceeds to the centrifuge or biomass removal.

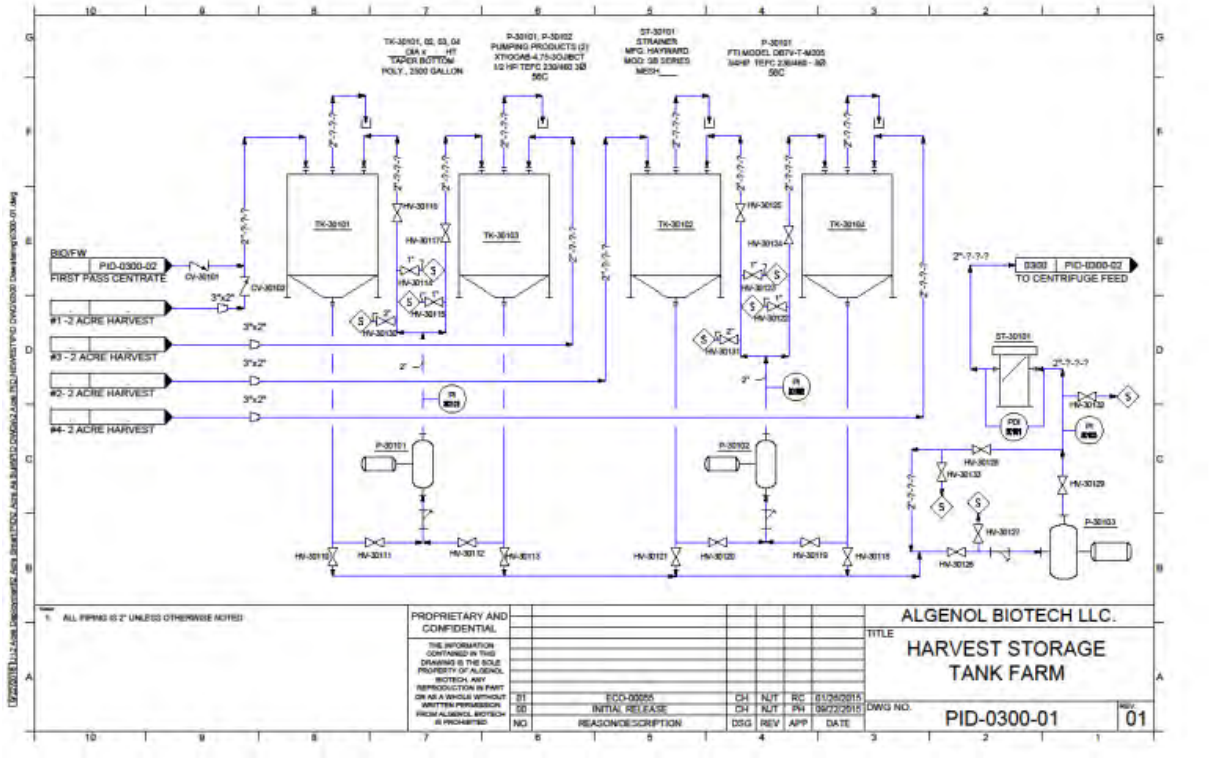


Figure A-35. Harvest storage tank farm PID.

Figure A-36 shows the PID for the external connections to the centrifuge. As noted, the centrifuge PID was developed and manufactured by GEA Westfalia (Figure A-37). The initial design intent contained within the biorefinery was to minimize the carbon loading in the feed stream of the Vapor Compression Steam Stripper. There also was potential for the biomass concentrate from this operation to be converted to biofuel intermediate in the Hydrothermal Liquefaction (HTL) unit. The centrifuge was commissioned during the summer of 2015, and evaluated with 9 harvests produced in VIPER 2.3 PBRs. In each case, the full harvest was run through the centrifuge, to provide enough volume to ensure performance analysis within a stable operating regime. Figure A-38 shows the efficiency of the centrifuge relative to the influent biomass loading (sOD) and flow rate. As can be seen from the figure, flow rates between 2.5 and 7.5 gallons per minute (GPM) produce similar biomass removal efficiencies across a range of incoming biomass densities.

Another important element of the centrifuge performance was the energy consumption. Figure A-39 shows the relationship between flow rate and energy per unit volume required for effective biomass separation. Several key conclusions were reached during the evaluation of centrifugation as a biomass separation technique:

1. Biomass separation efficiency is affected by flow rate, with efficiencies in the 75-95% range at 5 gpm, across a sOD range of 3-7 (biomass concentration of 1.5-3.5 g/L).
2. Using a second pass increased the recoveries to over 90% across a flow rate of up to 7.5 gallons per minute with energy consumption of no more than 1.54 kWh/m^3.
3. These conclusions provide important design guidance for a commercial scale system.

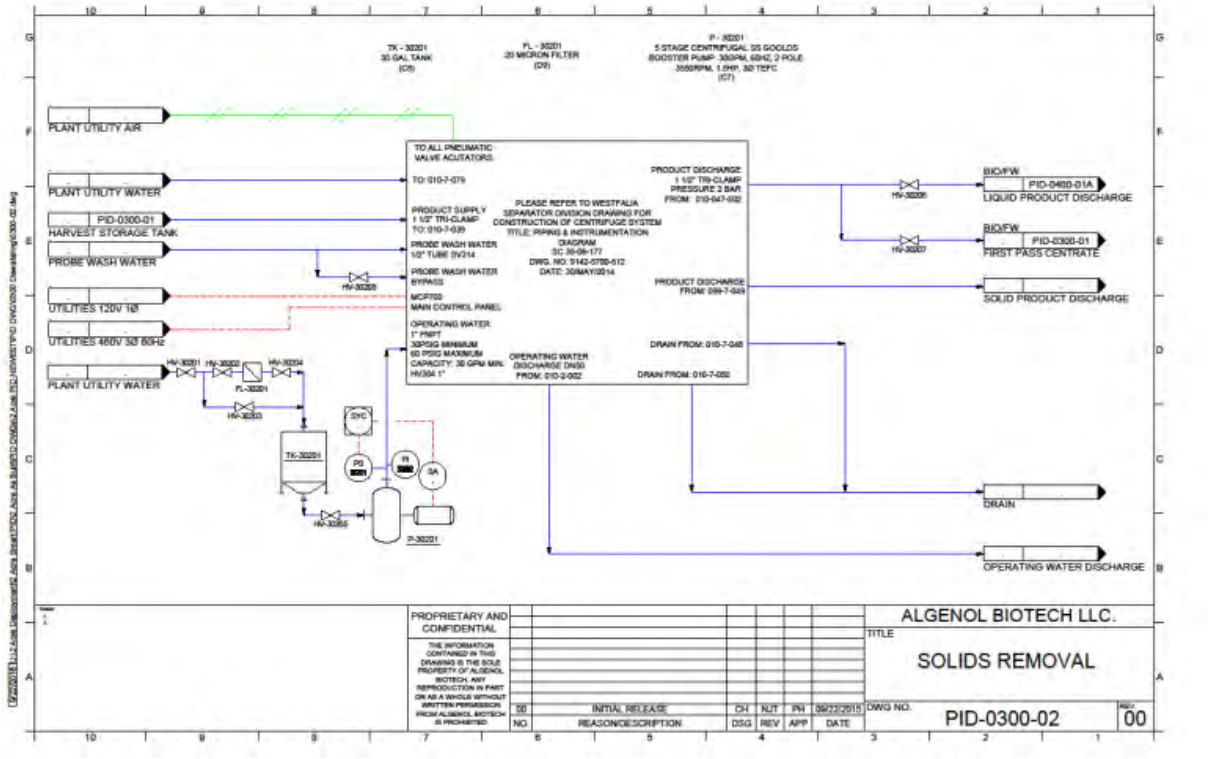


Figure A-36. Centrifuge external connection PID.

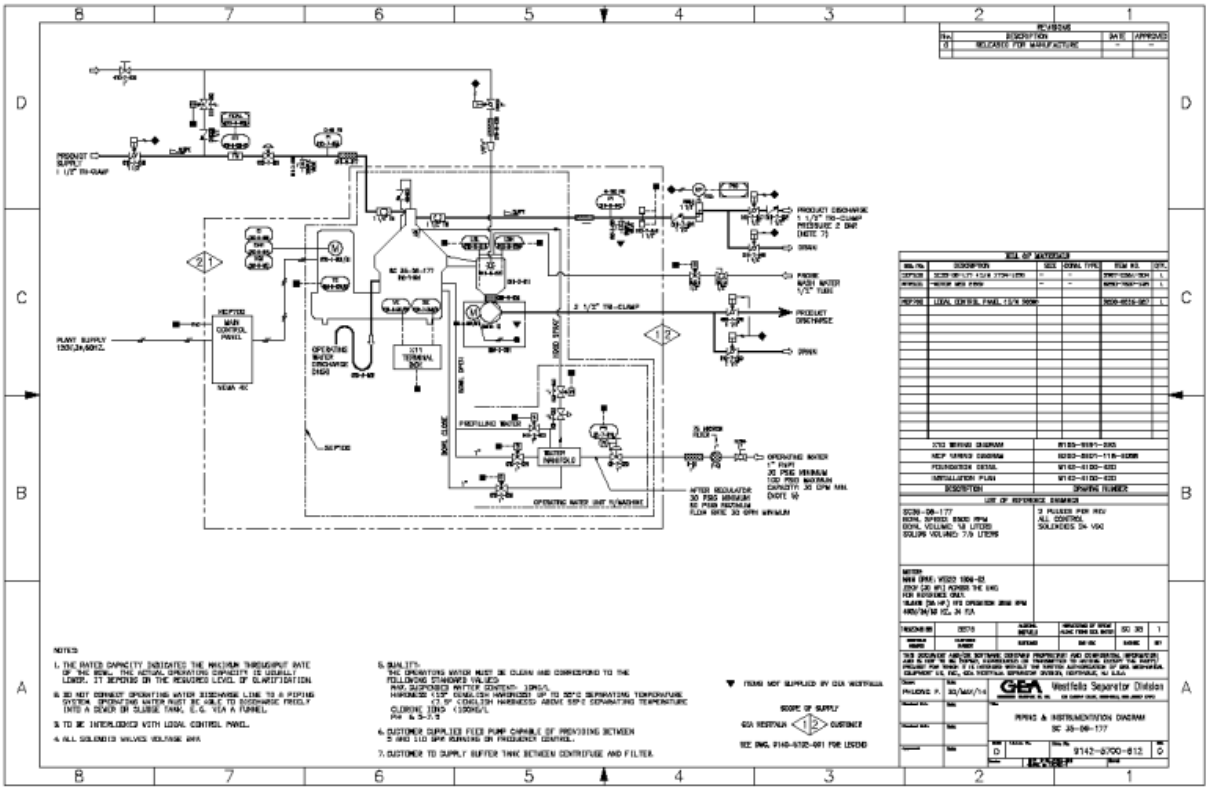


Figure A-37. GEA Westfalia SC-35 centrifuge PID.

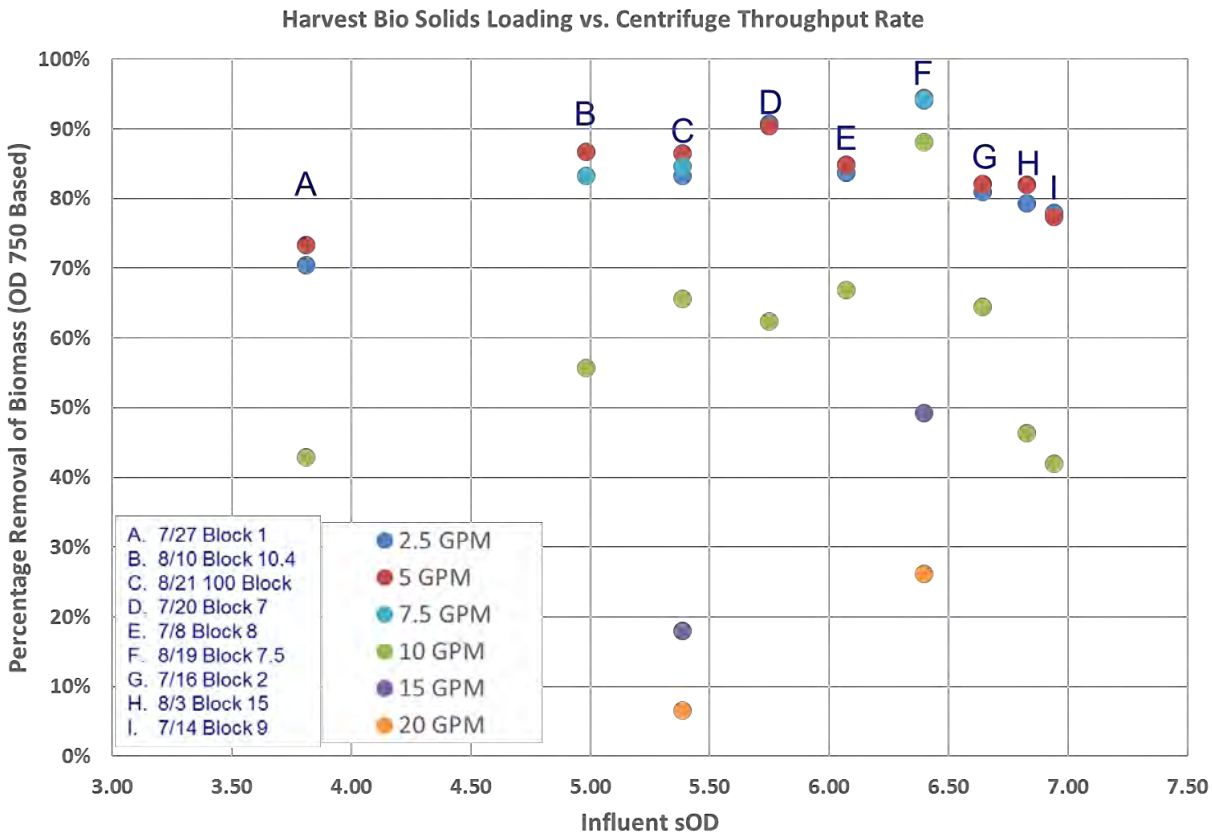


Figure A-38. SC-35 centrifuge performance with varying biomass loadings (sOD) and flow rates.

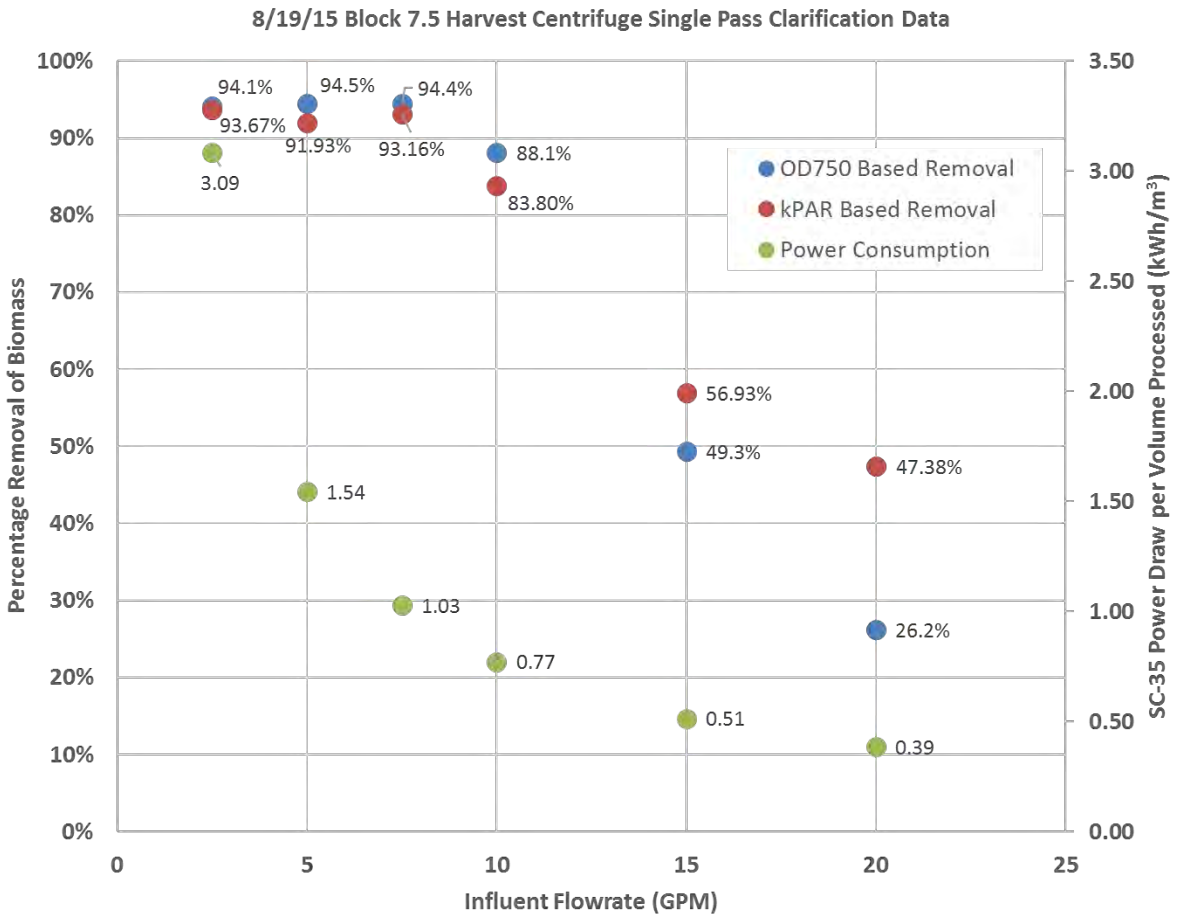


Figure A-39. SC-35 centrifuge energy and biomass removal efficiency using Algenol's ethanol producing cyanobacterium.

Ethanol processing

Ethanol separation systems design – This section describes the design basis for Algenol pilot plant Vapor Compression Steam Stripper (VCSS). The current VCSS is designed to process the culture from the1.8 acre IBR facility. Key design parameters are summarized in Table A-5. The purpose of the VCSS is to convert the salt water/ethanol mixture from a disk stack centrifuge in Biomass Dewatering to a 10x concentrated freshwater/ethanol mixture. The system is a highly heat integrated design which utilizes plate heat exchangers to recover sensible heat from the hot waste and product streams and a shell-and-tube kettle boiler to recover the latent heat from the vapor product (Figure A-40). Algenol's VCSS has three critical points of energy recovery: the feed/effluent heat exchanger, the boiler heat exchanger, and the product heat exchanger downstream of the boiler. The column and high temperature feed/waste piping are made of titanium for corrosion resistance to chloride pitting.

Table A-5. VCSS process design basis for Algenol IBR.

Parameter	Unit	Design	min	max	Remarks
Feed rate to VCSS	gpm	4.0	2.0	5.0	
Ethanol in feed	mass %	0.4	0.05	0.5	
Total solids	mass %	3.54	0.0		
Biomass content	mass %	0.04	0.0		Max unknown at

Parameter	Unit	Design	min	max	Remarks
					this time
Ash content	mass %	3.5	0.0		
Chloride	mass %	1.9	0.0		
Carbonate	mass %	0.015	0.0		
Calcium	mass %	0.060	0.0		
Magnesium	mass %	0.090	0.0		
Phosphate	mass %	0.0003	0.0		
Ammonium	mg/L	5	0.0		
Volatile organic sulfur compounds	mg/L	10	0.0		
Viscosity	cP				
Specific gravity		1.025			salt water
Thermal conductivity					primarily salt water, not determined for culture
Antifoam	mg/L	30			silicone free
Antiscalant	mg/L	50			
pH		7.5			
Feed storage tank	gal	10,000			2 x 5,000
Column temp.	°C	101			
Column press.	psig	0.9	0.5		
Column theoretical stages		15			
Reboiler feed/make-up					use RO FW

Figure A-40. Vapor Compression Steam Stripper (VCSS) at Algenol's IBR.

The feed to the VCSS from the centrate storage tank should have an OD reduction of 80% relative to the harvest OD. This corresponds to 80% removal of algae solids. The ethanol concentration should be between 0.1 to 0.5 %v/v to be qualified for the VCSS process. Under normal process conditions, the VCSS feed flow rate is 4 gallons per minute. The ethanol concentration after a single pass through the VCSS should increase by a factor of ~10. Partial recycle of the product to the feed stream can be done to increase the outlet concentration further, which is known as the multi-path VCSS process. The standard VCSS final product should have an ethanol concentration no less than 5%v/v before transferring to the stripper, rectifier and dehydrator (SRD) unit downstream of the VCSS. During steady state operation, the average product flow rate is at 0.4 gallons per minute.

Two main functions of VCSS are ethanol separation using a steam stripper, and latent heat recovery utilizing vapor compression technology. The VCSS process works through a tight heat integration and efficient utilization of energy. Ethanol losses are required to be less than 2% in the bottoms stream. The mechanical design is divided into liquid in and out and steam in and out areas. The control design is based on the control logics including transmitter feedback, transmitter interlocks, digital switches indication, digital switches on/off command and PID controls.

The dilute ethanol/salt water mixture from the post centrifuge storage area is pumped to the VCSS feed storage tank. The dilute mixture is pumped and pre-heated by waste discharge stream via a heat exchanger and preheater before entering the degasser, and then steam stripper. The steam stripper column concentrates the ethanol/freshwater mixture up to >5%v/v (depending on the inlet concentration) in the overhead stream, which is sent to the vapor compressor. The heat of compression from the vapor compressor is used to generate steam in the stripper reboiler. Steam is generated by condensing the 5%v/v ethanol/freshwater mixture in the stripper reboiler. Required excess heat is supplied by heaters. The condensed concentrated ethanol/salt water mixture is cooled in the heat exchanger. The cooled product is sent to the wet product storage tank.

The bottoms (primarily salt water) off of the steam stripping column is sent to the steam stripper economizer where the feed stream is heated. The cooled bottoms stream is sent water discharge.

VCSS process flow diagrams – Figure A-41 and Figure A-42 are the process flow diagrams for the VCSS as installed in the Biorefinery. The first shows the feed and take off support equipment, and the second shows the flows within the elements of the VCSS design.

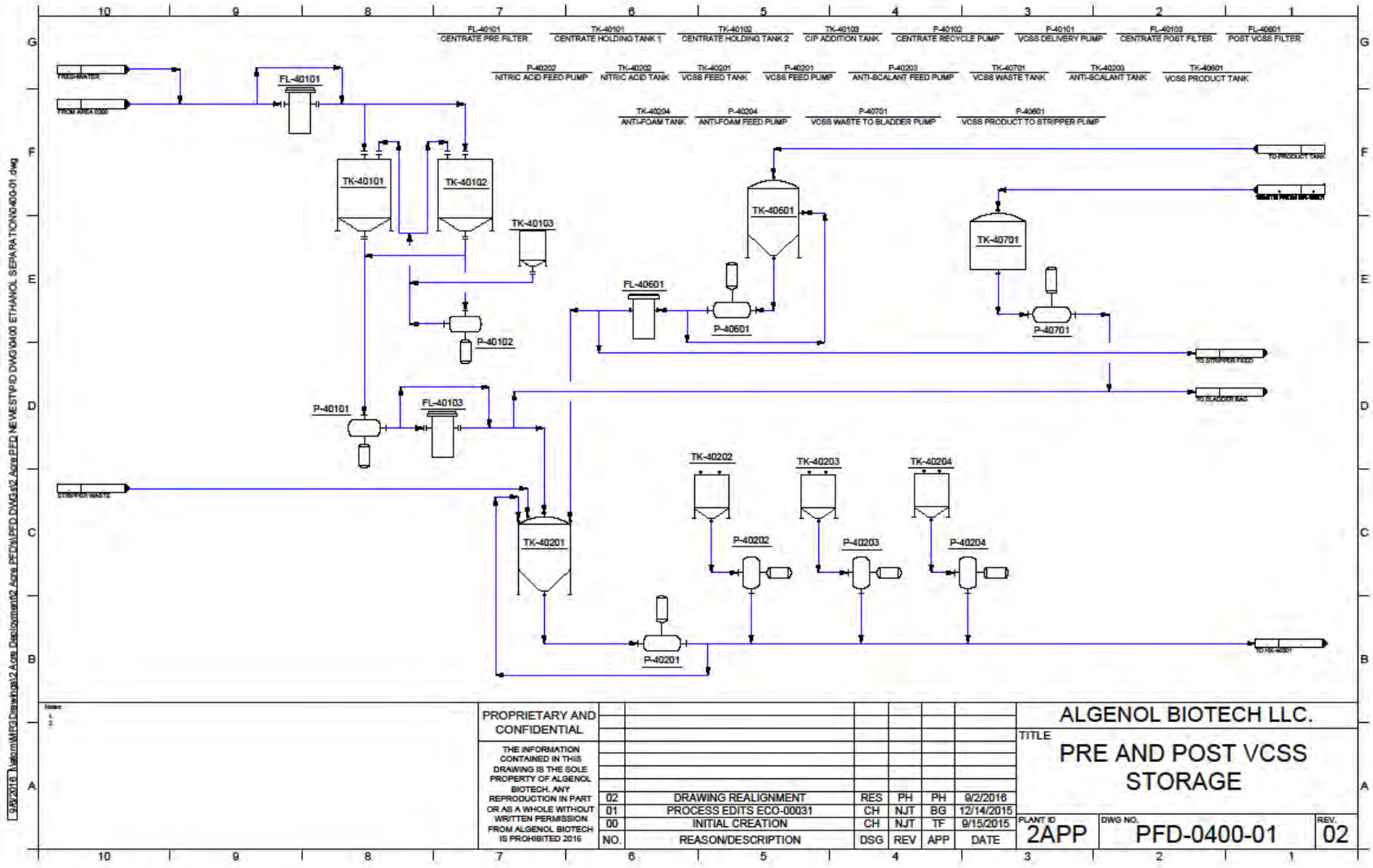


Figure A-41. VCSS feed and product storage PFD.

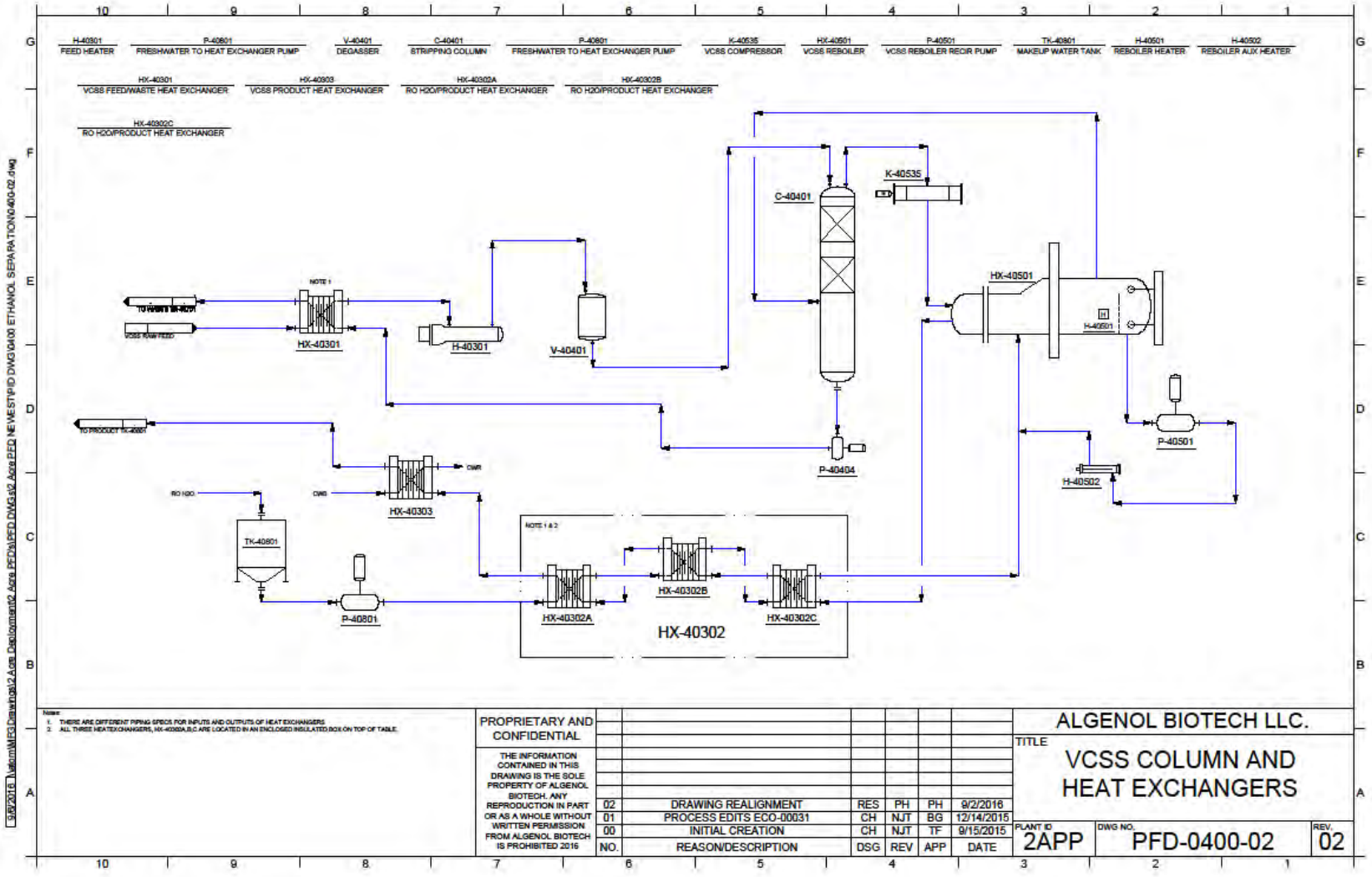


Figure A-42. VCSS column and heat exchangers PFD.

The VCSS is designed to enhance mass and heat transfers for low energy ethanol separation. The core VCSS physical connections are represented in detail in the VCSS PIDs. Mechanical design is separated as follows:

– *Steam stripper column process liquid inlet and outlet:* Centrate solid and ethanol contents are measured prior to the VCSS process. The centrate is pumped from the feed tank TK-40201 via feed pump P-40201. The feed tank reservoir starts at 600 gallons and a refill is required at 150 gallons. The liquid in or feed stream [cold] and liquid out or waste steam [hot] are countercurrent flow streams undergoing heat transfer via heat exchanger HX-40301. Preheater H-40301 supplies additional heat to warm up the feed to near boiling. Depending on valve position, feed liquid can run at one pass through the VCSS system from feed to waste, feed recirculation or feed discard modes. The waste tank only has one way in and out connections. Waste tank outlet is attached P-40601. During normal operation, the feed-in flow rate is 4 gallons per minute. Liquid in and out flow rates should have less than 0.5 gallons per minute differences.

– *Steam stripper column steam inlet and outlet:* Main heat transfer between steam-in or makeup RO water [cold] and steam-out or product [hot] occurs in the reboiler (HX-40501). Main reboiler heater (H-40501) should supply the additional heat to vaporize enough fresh water for stream stripping. Both main reboiler heater (H-40501) and reboiler start-up heater (H-40502) should run simultaneously to heat the reboiler reservoir RO water during start up. Depending on the valve position, product can be sent to SRD stripper if ethanol content is greater than 5 %v/v or recycle to feed tank to be re-concentrated. During normal operation, product and RO water makeup flow rates should match closely.

VCSS control design – Programmable logic controller (PLC) offers an I/O friendly and high speed control environment. The plant information is easily communicated to operators and engineers via National Instruments LabVIEW 2014 software, which runs on an 8-Slot National Instruments cRIO (or PLC) 9073. The input and output signals are delivered by transmitters and switches. All of the configuration settings are maintained in a local configuration file on the cRIO as well as a copy on a server share that the HMI (Human-Machine Interface). Any updated information is shared between PLC and HMI. Figure A-43 below is the VCSS main control diagram displaying the integrated VCSS system with critical control parameters for both start up and normal operation modes. The critical information in the main control page can be divided into digital transmitter readings, transmitter interlocked alarms, digital switch indications, digital switch I/O alarms & commands and major heaters & pumps on/off status and commends. Complicated PID controls, individual mechanical controls, hardware settings, transmitter parameters and completed PLC data outputs are in separated tabs. Finally, there are only two types of the control hardware in the VCSS system: 4-20 mA transmitters and digital switches.

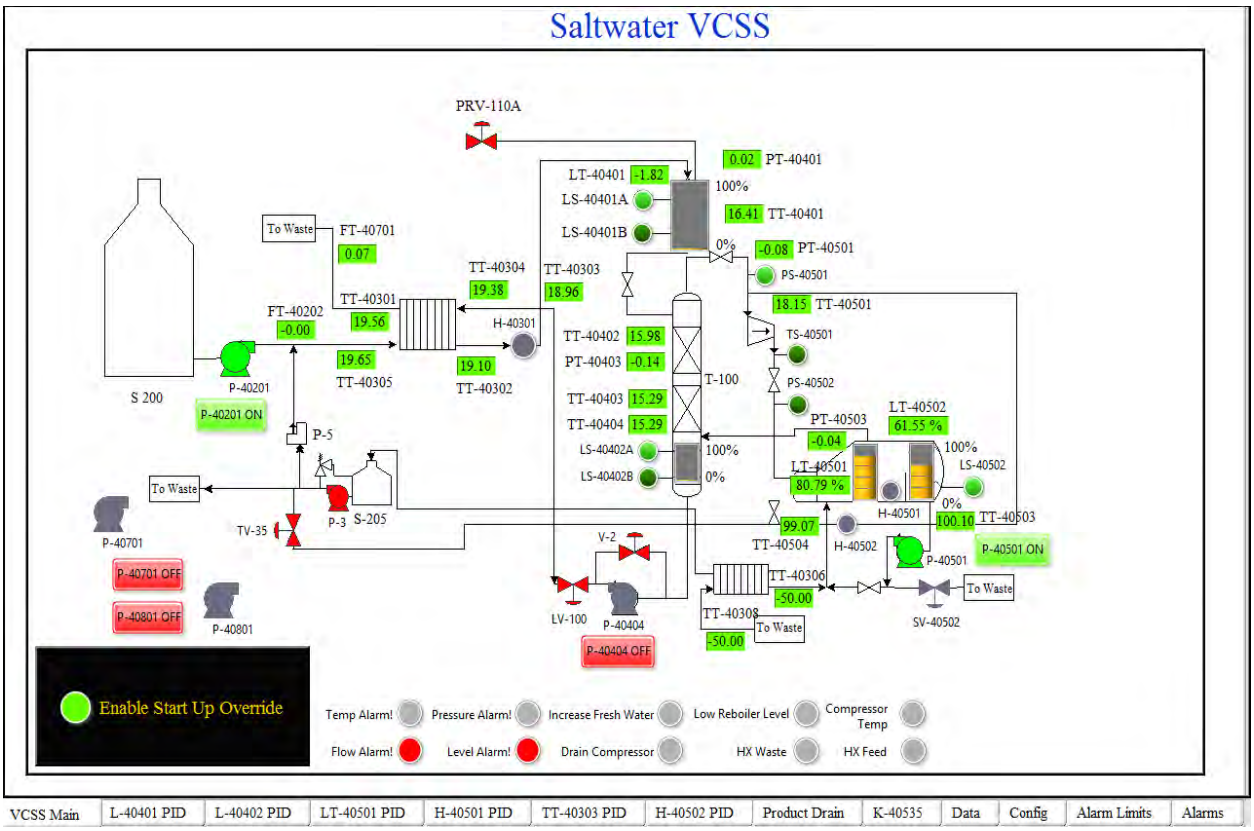


Figure A-43. VCSS PLC user interface screen.

VCSS performance – The water/ethanol stream effluent from the centrifugation process proceeds to the Vapor Compression Stream Stripper for concentration from the harvest ethanol percentage to a level of approximately 10 times the feed concentration. At the project inception, the intended harvest (VCSS Feed) concentration was to be 0.5 – 1.0 %v/v, with an intended effluent concentration of 5.0 to 10%v/v. These concentrations depended on multiple factors, including organism productivity, minimization of ethanol loss through venting of cultivation system, elimination of consumption by other organisms, and cultivation time between harvests. As the vertical PBR cultivation system challenges were addressed, the feed concentrations were slightly less than the original design targets. Table A-6 shows the run history and VCSS performance across a series of runs completed in 2016.

As can be seen from the table, the VCSS meets the design intent and exceeds a 10x concentration of the feed stream ethanol. Another key design element for the VCSS is energy consumption. Several simulations of the design were performed by Algenol, Georgia Tech and Linde showing that energy consumption can be greatly reduced by lowering the approach temperature. Validation of these predictions are difficult at small scale, however, due to substantial heat losses to the environment.

Table A-6. VCSS operational performance.

	Run dates	Feed	Bottom	Top	Top/Feed	EtOH loss in bottoms
		vol% EtOH	vol% EtOH	vol% EtOH	EtOH ratio	% lost
Block 14.3	Jan. 22,25,26	0.208	0.0050	2.33	11.2	2.4
Block 13.4	Mar. 10,11,14,15	0.047	0.0010	0.49	10.5	2.2
Block 14.4	Not harvested					
Block 13.5	Apr. 9,10	0.137	0.0020	1.41	10.4	1.5
Block 14.5	Apr. 13,14,15	0.259	0.0012	2.96	11.4	0.5
Block 13.6	May 4	0.237	0.0036	2.42	10.2	1.5
Block 14.6	May 7,8	0.279	0.0039	2.71	9.7	1.4
Block 13.7	May 29,30	0.322	0.0076	5.78	17.8	2.4
Block 14.7	June 1,2	0.355	0.0057	4.51	12.7	1.6
Block 13.8	June 23,26,28,29,30	0.295	0.0175	5.61	19.0	5.9
Block 14.8	June 30, July 1,2	0.342	0.0573	6.21	18.2	16.8
Block 14.9	July 23,24, 25	0.286	0.0229	5.07	17.7	8.0
Block 14.10	Aug, 16, 17	0.338	0.0002	4.21	12.5	0.1
Block 3.1	Aug, 19, 20	0.381	0.0044	4.97	13.0	1.2

Repetitive operation of the VCSS did, however, produce the design basis for the next generation system, which would meet the energy balance requirements. Table A-7 shows the analysis of the energy consumption measurements averaged across more than 5 runs. The current reboiler design was heat transfer area limited, which corresponds to about a 5kW loss, and an improperly designed product condenser resulted in a second 5 kW loss. Both of these issues would be simply rectified during the next scale design process.

Table A-7. Energy usage analysis of IBR VCSS and commercial scales.

	Algenol IBR-VCSS		**Algenol IBR Projection(2016)**		**TEM-P90**	
	2-acre	**2000-acre**	**Energy usage in VCSS 2000-acre**		**Energy usage in Ethanol Separation 2000-acre**	
	4.2 gpm Feed	2,410 gpm Feed	kwh/gal ethanol	MJ/MJ ethanol	kwh/gal ethanol	MJ/MJ ethanol
Electricity, KW	7.5	4,300	2.3	0.1	1.6	0.07
Heat, KW	24.5	14,060	7.6	0.34	5.0	0.23
Reboiler Heat, KW	16.7	9,585	5.2	(~75% latent heat recovery)	4.1	(80% latent heat recovery)
			Normalized to 7800 gepay		Normalized to 7800 gepay	

The final important element that affects the energy analysis is the lower than expected ethanol feed concentration. The design basis was for modelling was 0.6 to 3.0 %w/w, whereas,

due to challenges with the cultivation systems, the feed concentrations were typically in the 0.3 %w/w range.

Ethanol dehydration system design – The effluent product stream from the Vapor Compression Steam Stripper, 5–10% v/v ethanol, is then run through a standard Stripper, Rectifier and Dehydration (SRD) system to provide ethanol with 99.5 v/v% purity (Figure A-44). This product met all specifications for fuel grade ethanol as validated by ASTM testing.

The stripper was designed and built by Membrane Technology and Research (MTR) company. MTR was one of the original partners in the IBR project and was commissioned to supply a vapor permeation membrane unit for taking the VCSS output to fuel grade ethanol in a three stage operation. That unit was installed, but due to unreliable performance, it was decommissioned. Only the stripper continued in service. The feed stream contains 3–10% v/v ethanol (i.e., the product stream from the VCSS). The overhead (i.e., product effluent) containing about 40% v/v ethanol is transferred to the rectifier. A bottoms stream containing about 0.5% v/v ethanol is obtained which is recycled to the VCSS feed tank.

The rectifier takes the stripper product at about 40% v/v ethanol and produces an overhead product stream that is about 90% v/v ethanol and a bottoms stream containing about 5% v/v ethanol. The product stream is transferred downstream to the dehydration unit, while the bottoms are recycled upstream to the stripper.

The molecular sieve dehydrator uses pressure swing adsorption which is the industry standard for ethanol dehydration. The feed from the rectifier contains about 90% v/v ethanol and the product contains 99.5% v/v ethanol. The molecular sieve columns are regenerated with an ethanol sweep stream. The sweep stream generates a 50% v/v ethanol in water stream that is recycled back to the rectifier feed tank.

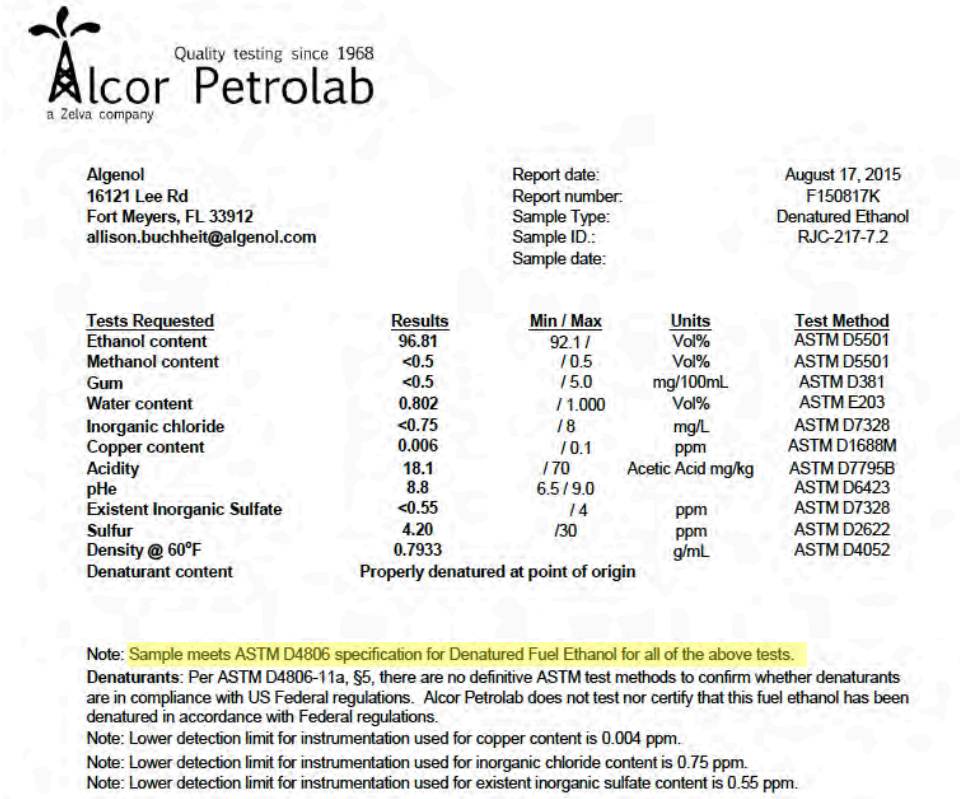
The SRD performance was assessed through the processing of about 15 batches, and successfully produced fuel grade ethanol > 99.5 v/v% ethanol. All of the technology post-VCSS is standard industry practice. There is significant potential for improvements, e.g., more extensive heat integration. Demonstration of these improvements would be a key part of further advancement of the technology.

Figure A-44. SRD unit at Algenol's IBR.

Ethanol separation processes conclusion – The goals of the Integrated Biorefinery project included the design of the infrastructure to support delivery of the harvest from the cultivation field to the ethanol refinement area, and to separate and concentrate the ethanol, resulting in collection of 99.5 v/v% ethanol. In August 2015, Algenol received a fuel-grade analysis from an independent laboratory, which certified Direct-to-Ethanol® as capable of producing fuel-grade production derived from cyanobacteria (Figure A-45). Below is a summary of successes demonstrated during integrated operation of the ethanol extraction systems at the IBR.

1. Harvest from the vertical PBR system is transferred to storage tanks in the refinery.
2. The material in the storage tanks is dewatered through the SC-35 Centrifuge, with the biomass reserved for future HTL processing and the clarified liquid being sent to the VCSS
3. The VCSS concentrated the ethanol stream by approximately 10x, which met the design intent.
4. The SRD unit successfully produced 99.5 v/v% fuel-grade ethanol.
5. All of the infrastructure needed to support these activities has been designed, installed and operated at the Fort Myers site.

After challenges were addressed with the feed stream production, multiple batches were processed under steady state conditions, from which the operational and design basis data to inform the development of commercial scale versions of all of this equipment has been acquired.



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16121 Lee Rd
Fort Meyers, FL 33912
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Report date: August 17, 2015
Report number: F150817K
Sample Type: Denatured Ethanol
Sample ID.: RJC-217-7.2
Sample date:

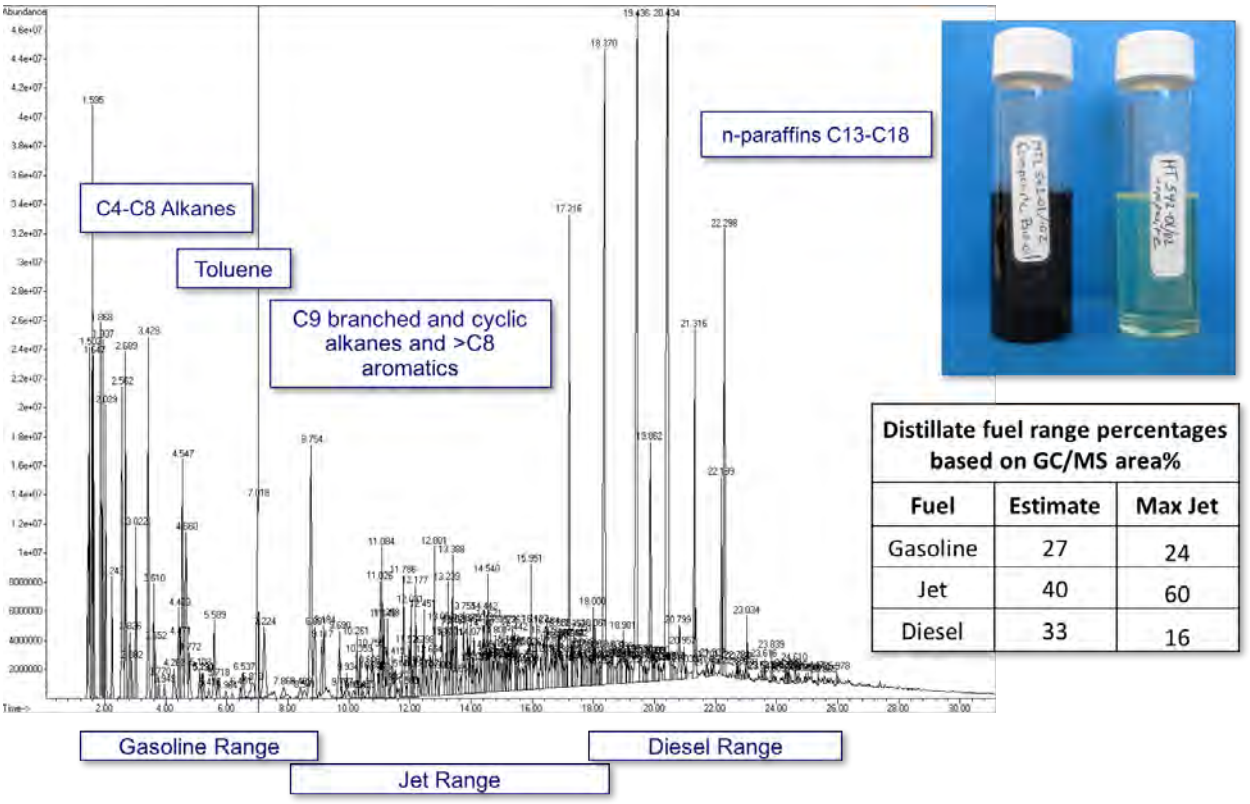
Tests Requested	Results	Min / Max	Units	Test Method
Ethanol content	96.81	92.1 /	Vol%	ASTM D5501
Methanol content	<0.5	/ 0.5	Vol%	ASTM D5501
Gum	<0.5	/ 5.0	mg/100mL	ASTM D381
Water content	0.802	/ 1.000	Vol%	ASTM E203
Inorganic chloride	<0.75	/ 8	mg/L	ASTM D7328
Copper content	0.006	/ 0.1	ppm	ASTM D1688M
Acidity	18.1	/ 70	Acetic Acid mg/kg	ASTM D7795B
pHe	8.8	6.5 / 9.0		ASTM D6423
Existent Inorganic Sulfate	<0.55	/ 4	ppm	ASTM D7328
Sulfur	4.20	/30	ppm	ASTM D2622
Density @ 60°F	0.7933		g/mL	ASTM D4052
Denaturant content	Properly denatured at point of origin			

Note: Sample meets ASTM D4806 specification for Denatured Fuel Ethanol for all of the above tests.
Denaturants: Per ASTM D4806-11a, §5, there are no definitive ASTM test methods to confirm whether denaturants are in compliance with US Federal regulations. Alcor Petrolab does not test nor certify that this fuel ethanol has been denatured in accordance with Federal regulations.
Note: Lower detection limit for instrumentation used for copper content is 0.004 ppm.
Note: Lower detection limit for instrumentation used for inorganic chloride content is 0.75 ppm.
Note: Lower detection limit for instrumentation used for existent inorganic sulfate content is 0.55 ppm.

Figure A-45. Fuel-grade ethanol certification from cyanobacteria-produced ethanol made at the Algenol IBR in 2015. The sample sent for analysis was denatured with 2% gasoline prior to submission. The product complied with ASTM D4806 standards.

Biomass processing

During the course of the project, the operating paradigm of the cultivation field changed in concert with the PBR design which necessitated a batch production model. This introduced the potential to convert the biomass stream, post centrifugation, to a biocrude intermediate. A summary of the Algenol funded work with Pacific Northwest National Lab (PNNL), shown in Figure A-46, indicated that up to 70% of the carbon in the biomass feed stream could be converted to biocrude intermediate with 34% efficiency using a Hydrothermal Liquefaction (HTL) process. The composition of the result crude contained hydrocarbon oils with characteristic compositions similar to those found in gasoline, jet and diesel fuels. While this work and the partnership with PNNL are outside the scope of this project (as originally formulated), a pilot scale HTL unit was installed (but not yet commissioned) in the Fort Myers Biorefinery.



Distillate fuel range percentages based on GC/MS area%		
Fuel	Estimate	Max Jet
Gasoline	27	24
Jet	40	60
Diesel	33	16

Figure A-46. Hydrothermal Liquefaction process summary based on work performed by PNNL on AB1 biomass supplied by Algenol. As indicated, this work was funded by Algenol separately.

Water treatment

The raw water source for cultivation medium was a salt water aquifer below the Fort Myers facility. The well was drilled and commissioned in April 2013 at 1,600–1,800 ft depth and supplied raw salt water to the IBR and PDU. All wells were operated in compliance with Florida Department of Environmental Protection, and a monitoring program was established to support regulatory compliance.

Clean and sanitized cultivation waters and PBR systems are both necessary for successful and repeatable ethanol production batches. The initial water treatment design for the 40, 400, and 4,000 Block required ultrafiltration (GE Spiral Membrane Ultra Filtration System; 50 gpm) followed by ozonation of the salt water prior to distribution to the cultivation PBRs. Ozonation resulted in a high level of total residual oxidants (TRO) in the salt water, and the highly oxidative water was effective at killing bacteria and contaminants remaining after ultrafiltration. Residual oxidants in the water remained active for several days after treatment and helped to sanitize piping and PBRs prior to culture inoculation. Nevertheless, decay of ozone oxidants in salt water was difficult to control. If the salt water medium was reactive at inoculation, initial culture growth lagged, and total downtime for cleaning between batches varied from three days to over a week, depending on ambient temperature. Finally, gaseous and liquid ozone sterilants decreased overall plastic life of PBR films and fitments.

For the 1.8-acre production field, ozone treatment was replaced with a more comprehensive clean-in-place (CIP) approach that relied on chemical caustic and acid treatments. In this case, salt water medium was simply micro-filtered in the system after the system piping and plastic PBRs were sanitized with CIP. Final salt water treatment design specifications were:

1. Salt water well
2. Holding tank
3. 1.0 µm filtration (dead end)
4. 0.2 µm filtration (dead end)
5. Co-injection of salt water, concentrated nutrient solutions, and culture inoculum.

An oxygenation step was contemplated at the holding tank stage to reduce dissolved sulfides. This step is recommended if the salt water source has high dissolved sulfides as the reduced water interferes with consistent dissolution of trace nutrient salts. Regular and consistent operation requires routine integrity monitoring of the microfiltration systems.

Make-up water

Algenol's original concept with the horizontal photobioreactor was to collect the ethanol-water condensate that formed on the inside of the PBR headspace (a solar still analog) and then to purify ethanol from the freshwater solution in the VCSS. In this case, freshwater recovered from the VCSS would need to be filtered, sterilized and transported back to the PBR to avoid increases in culture salinity.

With different physical headspace attributes and daily temperature characteristics, condensate collection is impractical in current vertical PBR designs. Therefore, the VCSS was reconfigured to process ethanol from the salt water culture. Commercially, it is conceivable to recycle salt water remaining in the VCSS bottoms. The media recycle process would include water treatment steps to reduce dissolved organics and filtration to remove bacteria. Water treatment costs would have to be balanced against simply using new salt water for culture activities and would be driven in part from costs associated with acquiring and transporting new salt water from source. With a local salt water well at the IBR, culture medium was always made using new salt water.

Nutrient supply, delivery systems and preliminary design of control systems for the photobioreactors and integrated biorefinery

The objectives of the cultivation system control and data acquisition system were 1) to monitor key parameters pertinent to understanding general operation, production and material balances and 2) to automatically deliver phosphate nutrient solution and control CO_2 injection. Examples of the former are monitoring sensors such as culture pH and temperature and sensors that support mass flow, concentration and pressures of the photobioreactor gas system. Both monitoring and controlling functions integrated into the IBR SCADA system stored data in Algenol's data historian 'Kyanos' (Figure A-47).

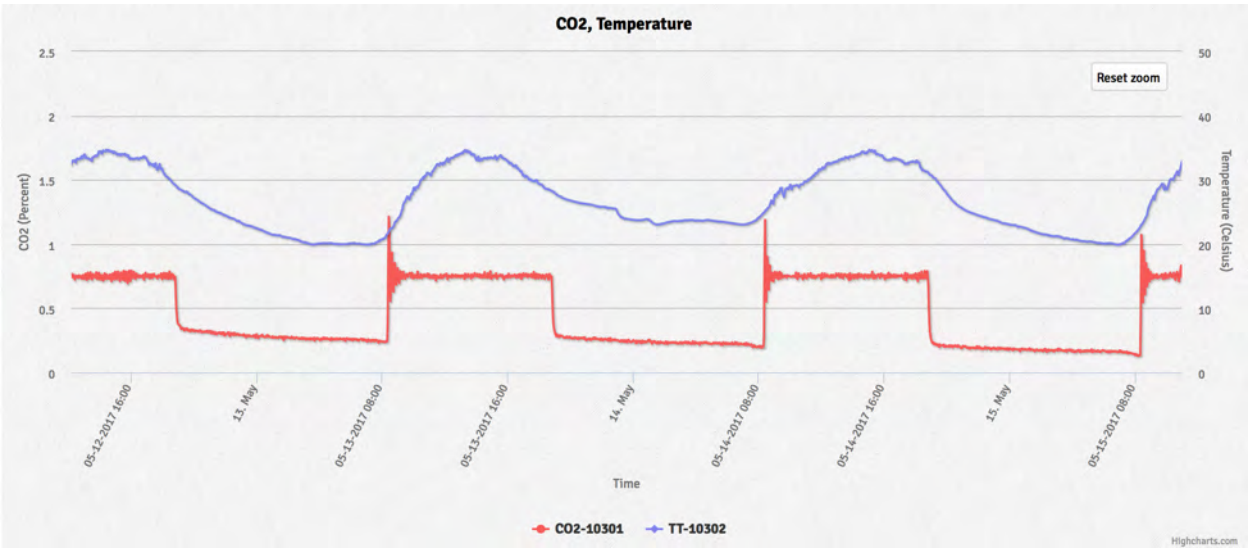


Figure A-47. Kyanos user interface and screen output example of real-time temperature and CO_2 concentration data visualization from IBR photobioreactor operation monitoring.

Kyanos is Algenol's repository for online computer monitored data from laboratory, PDU and IBR experiments and operations. In addition, many analytical data points associated with manual PBR sampling and processing are also directly stored in the database. Data in Kyanos are backed up to a remote location daily. Kyanos also provides a user interface to visualize real-time data from the IBR and other computer monitored systems. Since Kyanos is networked, it can be accessed securely from remote locations, and the system provides automated alarms and alerts to operators if key performance metrics fall outside design parameters.

In addition to data monitoring, an ethanol production SCADA system has three main automated computer operations:

1. Phosphate delivery – Phosphate is delivered daily from a tank with a known concentration of phosphoric acid. The logic determines the length of time the distribution pump is operated to deliver the required daily phosphate amount.
2. CO_2 delivery – a common commercial method to maintain dissolved CO_2 in the culture medium and to support photosynthetic carbon uptake and ethanol production is to monitor culture pH and add CO_2 to maintain the pH in a narrow range.
3. Gas recycle – ethanol photobioreactors require bubbling with a CO_2/air mix to homogenize the cultures, to deliver CO_2 to the system, and to remove oxygen from the system. The bubbling operation also strips vaporized ethanol, and the outflow gases still have a relatively high CO_2 concentration. To reduce CO_2 and ethanol losses, the Algenol PBR air system is closed, and cultivation gases are recycled (Figure A-48).

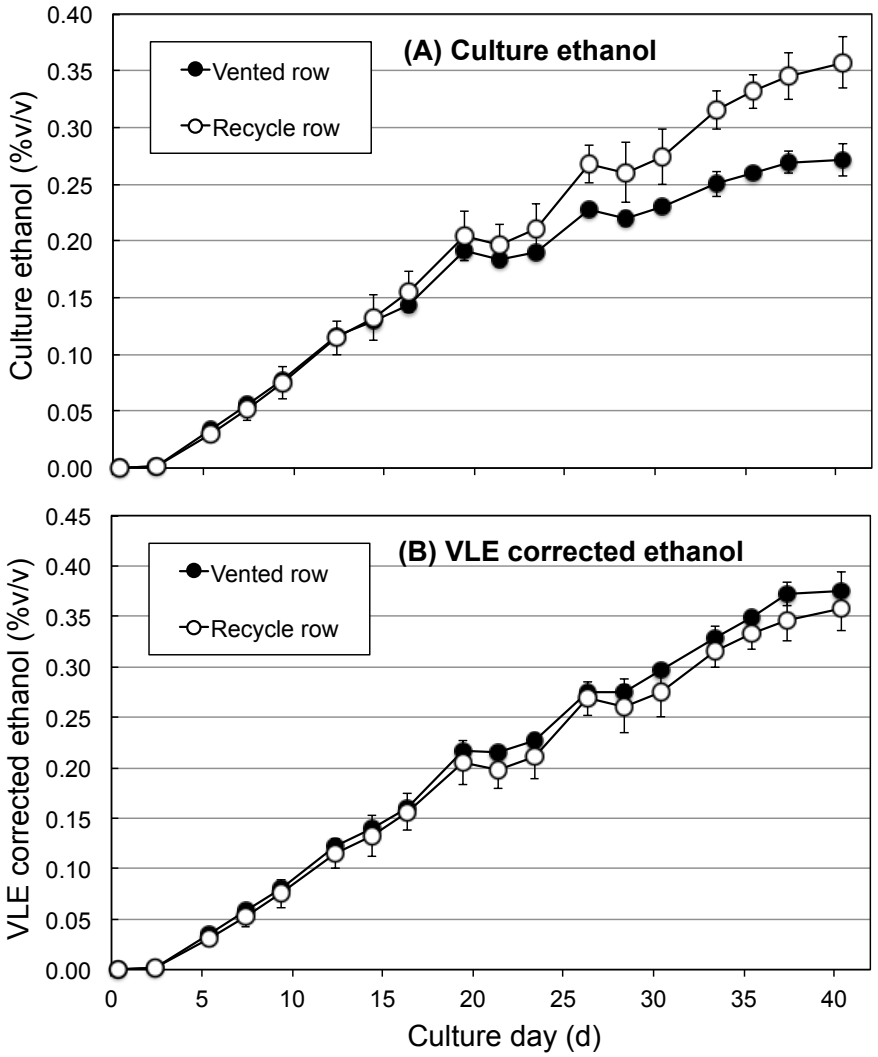


Figure A-48. Culture ethanol concentration in outdoor vertical photobioreactors operated in gas recycle mode and venting mode. Ethanol losses in the vented treatment were corrected in (B) for theoretical losses estimated via a vapor liquid equilibrium model, and a comparison between treatments demonstrated little overall loss in productivity in the gas recycling treatment.

Flue gas supply

Flue gas from both natural gas and coal-fired power plants is a potentially inexpensive source of carbon for industrial production of algal-derived biofuels. Algenol worked with a large Florida power producer to evaluate production of ethanol and biomass using flue gas as the primary source of CO_2 supplied to the algae at lab scale. These experiments were conducted under standard operating conditions designed to replicate annual average outdoor conditions in Fort Myers, Florida with regard to temperature and light. Productivities for flue gas treatments were compared to controls using diluted pure CO_2 as a carbon source. The observed biomass and ethanol productivity were comparable to those for outdoor cultivations for both the flue gas treatments and the pure CO_2 controls. Technical reports for both natural gas and coal-fired power plant flue gas are attached and a summary of each experiment follows.

For the natural gas-fired study, flue gas was collected from a natural gas-fired combined cycle unit at a Florida power station. Compositional analysis of the flue gas indicated that potential contaminants (CO, sulfides, mercaptans, NO_x, SO_x, and hydrocarbons) were all below

the limits of quantitation and the flue gas contained ~4.0% CO_2 and oxygen content was approximately 33% lower than atmospheric levels of oxygen. Lab-scale cultivations with AB0005 indicated there were no adverse effects from delivering carbon in the form of undiluted flue gas on either ethanol or biomass productivity. Cultures supplied with flue gas produced the same amount of biomass and ethanol as control cultures supplied with pure CO_2. No adverse effects of natural gas power plant derived CO_2 on Algenol's Direct to Ethanol® process were observed from a biological perspective. The relatively low concentration of CO_2 in natural gas derived flue gas requires supplementation during peak periods of productivity to meet the carbon demand of the algae.

For the coal-fired study, flue gas was collected from a coal-fired steam unit at a Florida power station. Unlike flue gas from natural gas power plants, coal flue gas has a CO_2 concentration of ~10-12%, sufficient to meet peak demands of ethanologenic cultures. However, burning coal may result in a gas stream with elevated levels of heavy metals, NO_x, and SO_X, potentially inhibiting production of biomass and ethanol. This may be exacerbated by the concentrating effect of gas recycling in photobioreactors, and thus the goal of this study was to evaluate the effects of a coal flue gas CO_2 supply compared to pure CO_2 in both single-pass, blow-through mode and in gas recycle mode. Compositional analysis indicated that many of the potential contaminants were likely removed in condensate traps during the collection process and the potential negative impact was minimized. The results of this experiment indicated that cultures supplied with coal flue gas as the CO_2 source had the same productivity over 28 days as culture supplied with pure CO_2 in both blow-through and recycle modes.

Subtask A.5 Architect search, and site selection, design and construction planning, employee recruiting, SOP development

Completed July 2011

Site selection

At the time of the application, Algenol had two separate sites that were to be evaluated for the IBR. The minimum size of property for the IBR was 25 acres. The site needed to be relatively flat and not located in a flood zone. The site also needed access to salt water, electricity, potable water, and road, sewer and phone/internet services.

Freeport, Texas Site – The Texas site option was located near Freeport, Texas in Brazoria County on land owned by Dow. Freeport is located along the southeastern Texas Gulf Coast approximately four miles inland from the Gulf of Mexico. The site is located adjacent (north) to the Dow Chemical Oyster Creek Plant. The site consisted of 26 acres of undeveloped grassland north of State Highway (SH) 332, approximately 0.7 miles northwest of the intersection of SH 332 and Farms-to-Market 523. A private access road borders the western boundary of the site and connects to SH 332. The entire site is a coastal prairie that is periodically harvested for hay to be used as cattle feed. The site had access to electricity and internet, but did not have access to city water, salt water or sewage services. Potable water, salt water and waste water services were to be provided via service trucks by Dow from their facilities at Oyster Creek. Sewage services were going to be provided by local sewage contractors. The Dow Texas site was ruled out during the pre-NEPA evaluation by Dow. Therefore, Algenol shifted all of its efforts to the Fort Myers, Florida site.

Fort Myers, Florida Site – The Fort Myers project site is located (26°30"' 23.15" N, 81°48' 43.79" W) in unincorporated Lee County, Florida approximately ten miles south of the City of Fort Myers (pop. 70,918) and less than a mile north of the town of San Carlos Park (pop. 26,800). The site is approximately three miles southwest of Southwest Florida International Airport. Lee County is on the southwest coast of Florida on the Gulf of Mexico (Figure A-49).

Figure A-49. Algenol IBR site in Fort Myers, FL.

The site consists of approximately 36 acres of leased land. The site is not located in a FEMA designated flood zone (Zone X flood designation). The property is fairly isolated from the general public and sits at the end of a dead-end road in a sparsely developed industrial area. The undeveloped land around the site is currently used for cattle farming. The closest residential area to the site is approximately 0.3 miles to the north on Fiddlesticks Boulevard and is separated from the site by a storm water canal and area of vegetation. The site is zoned for heavy industrial use and for chemical and allied products and manufacturing.

Electrical service is supplied by Florida Power and Light via above ground power poles that run down the west side of Lee Road. Three-phase power is available at the site. Telecommunications is supplied to the site by multiple providers of high-speed internet through multiple underground high-capacity fiber optic cables. Potable water is available to the site and is supplied by Lee County Utilities via a water main that runs down the west side of Lee Road. There is a 12” fire main that is located at the business park that is adjacent to the site. Site sewage treatment was supplied by Lee County Utilities via a sanitary line connected to an existing sewage pipeline on the Lee Road right-of-way for sanitary wastewater. Trash disposal and recycling is provided by multiple contractors under contract, and there are several licensed hazardous waste contractors in the area.

The Project site has car and truck access via Lee Road. Lee Road runs along the western side of the property. Lee Road intersects Alico Road approximately 1 mile from the site. Alico Road has an entrance/exit to Interstate-75 one and one-half miles east of the site.

Design and construction planning

The project team detailed the execution of the project and divided up each project task to identify the different areas that require design and construction efforts. Then the Team identified which portions of the project will be performed by an EPC, an architect, or internal design by Algenol engineers (Table A-8).

Table A-8. Project performance responsibilities.

Task/Equipment	Responsible Entity	Type of Contract
Upstream (Photobioreactor field)	Algenol engineers	N/A
Main building	Local architect	Fixed price
Process pavilion	Local architect	Fixed price
Process water system	EPC	Fixed price
Water well	Environmental consulting firm	Fixed price
Piping system	EPC	Fixed price
Downstream processing	Algenol engineers	N/A
Infrastructure site plan	Civil engineering firm	Fixed price

The team developed detailed task lists and associated timelines for each of the construction tasks to identify the duration of each task and the party responsible for completing the task. The primary risks to the timeline were in the upstream and downstream process areas that were to be completed by Algenol engineers. Since the upstream and downstream processes were under various stages of development for deployment at the IBR, the team first focused on the mass balance of the system to provide the information necessary to complete various design functions being completed by outside contractors. Once mass balances, site concepts, photobioreactor counts, energy and utilities estimates were detailed, the information was turned over to the design team to develop site lay-outs at the Texas and Florida sites and further planning efforts.

As the photobioreactor and culture systems designs and ethanol purification processes were continually developed and tested, the team recalculated the cost of construction and refined the methods that will be used to construct the Integrated Pilot-Scale Biorefinery. Algenol has a high-bay testing lab and a 4.5 acre outdoor Process Development Unit (PDU) in Fort Myers, FL. All piloting of the upstream and downstream process were done at these facilities. The team designed, constructed, installed and tested photobioreactors and evaluated various methods for the overall installation. Extensive work was completed in ordering and evaluating various materials that will be used at the IBR. The company's pilot scale Vapor Compression Steam Stripper was operated in various modes to inform the design of the larger unit to be located at the IBR. The team also conducted detailed engineering for the design of the CO_2 distribution system.

EPC and architect search

We evaluated several firms for the project. The following information was used for the assessment of criteria for pre-qualification and eventual bid evaluation (1) Financial standing and financial stability. (2) Management capability and systems, past performance and completed project quality, project management system, quality control policy, quality management system, experience of technical personnel, and management knowledge of biofuels. (3) Technical ability, such as experience in biofuels or related industry, applicable equipment and personnel. (4) Experience in similar projects regarding type and size (5) senior management's willingness to perform the work and the "fit" of the contractors' personnel to work with our project team.

For many of the firms we interviewed, the size of the project was not a good fit for their organization or they did not have an interest in working in this field. Since this was a first-of-a-kind project, many firms did not want to take the time, effort and risk to work on the project.

Employee recruiting

In planning the project, Algenol planned to hire a plant manager, a plant engineer and a project construction manager. The plant manager and plant engineer would be needed if the plant was to run continuously for an extended period of time beyond the length of the DOE project. Early in the project, it was decided that existing Algenol personnel would be used to staff the IBR until such time that additional staff were needed to operate the facility.

Standard Operating Procedure (SOP) development

The Process Development Unit in Florida was used to develop and test the production and laboratory equipment, protocols and procedures for the safe and efficient operation of the Biorefinery. All management, laboratory and operations personnel were trained at this facility. Once Biorefinery personnel were thoroughly familiar with the operation and maintenance of the PDU equipment and systems, they worked with PDU laboratory, operation and maintenance personnel to develop SOPs for the Biorefinery. PDU SOPs were used as a template for the development of SOPs applicable to the specific equipment, systems and operations of the Biorefinery. Biorefinery personnel implemented and tested the developed SOPs during the final system checks and shake down runs at the Biorefinery. PDU personnel consulted with Biorefinery personnel on a continual basis to ensure all process and safety improvements are implemented at the Biorefinery.

Part of the SOP development was a hazard and operability study (HAZOP) for all applicable equipment and processes. The HAZOP team worked to identify potential deviations from the documented design parameters. For all deviations, the team identified causes and the likely consequences and then decided whether the existing safeguards were sufficient. If not, the HAZOP team devised a corrective action list to install additional safeguards as necessary to reduce the risks to an acceptable level.

Once the draft SOPs were developed for each process and equipment, Management of Change (MOC) rules were implemented to manage all changes in the process and related SOPs. The MOC had a review and authorization process for evaluating proposed changes to facility design, operations, control, organization, or activities prior to implementation to ensure no unforeseen hazards were introduced into the operation and that the risk of existing hazards to personnel, the public in general, or the environment was not unknowingly increased. The MOC also included detailed steps to help ensure that potentially affected personnel were notified of the change and that related documents, such as SOPs, safety protocols and knowledge, control logic and so forth, were kept current.

Failures or events in processes and equipment that were identified were evaluated using Root Cause Analysis (RCA) methodology to identify and correct the root causes of the failures or events. The RCA process focuses on correction of root causes and has the goal of entirely preventing problem recurrence. Even though RCA is typically used as a reactive method of identifying the event causes, revealing the actual problems and solving them, Algenol commonly uses the methodology to preempt potential failures or events.

Subtask A.6 NEPA requirements

Completed July 2011

Algenol completed an Environmental Assessment (EA) for the Project in compliance with the National Environmental Policy Act of 1969, as amended (NEPA; 42 U.S.C. §§ 4321, et seq.); Council on Environmental Quality (CEQ) regulations (40 CFR Parts 1500 to 1508); and DOE NEPA implementing procedures (10 CFR Part 1021). Algenol prepared the EA in collaboration with professional environmental consultants, Potomac Hudson Engineers, and DOE's NEPA

compliance staff to evaluate the potential environmental consequences of constructing and operating the biorefinery. The EA considered two sites that were under evaluation at the time, one in Freeport, TX and the other in Fort Myers, FL that was ultimately selected for the Project because it offered a better environmental profile in addition to other business considerations.

Published in December 2010, the EA details the proposed action and no-action alternatives, identifies the existing environmental conditions of the site and analyzes potential site specific impacts on the environment that could result from construction and operation of a biorefinery. Impacts on the following resources were scrutinized in their individual context as well as from a cumulative impact perspective: land use; visual and aesthetic resources; cultural resources; air quality; noise; geology and soils; water resources; biological resources; waste management and hazardous materials; utilities and energy; transportation and traffic; public and occupational health and safety; socioeconomics; and environmental justice. The completed EA fulfilled Algenol and DOE's obligation under NEPA. Based on the information presented in the EA, DOE determined that authorizing expenditure of federal funds to support the biorefinery would not constitute a major federal action significantly affecting the quality of the human or natural environment as defined by NEPA. Accordingly, DOE's Office of NEPA Policy and Compliance issued a Finding of No Significant Impact (FONSI) on December 28, 2010 allowing the project to proceed to fruition.

Subtask A.7 Regulatory submission and approval

Completed July 2011

An important milestone achieved with the Environmental Protection Agency (EPA) during the Project was the approval of Algenol's pathway petition (Table A-9). In July of 2014, Algenol petitioned EPA for approval of a pathway for the generation of advanced biofuel Renewable Identification Numbers (RINs) consistent with the requirements of the Renewable Fuel Standard program. The new pathway would cover ethanol produced by photosynthetic cyanobacteria using Algenol's proprietary Direct to Ethanol® process. In the petition Algenol also described its ability and intent to produce a bio-oil co-product and took the position that an appropriate pathway already exists that will allow the Company to generate corresponding RINs. On December 2, 2014 EPA issued an approval of the requested new pathway and agreed, in writing, with Algenol's assertion that an appropriate pathway exists for the oil it will produce along with ethanol. As required by statute, EPA conducted a life cycle analysis of Algenol's process and stated in their approval documentation that ethanol produced through the Algenol pathway reduces lifecycle greenhouse gas emissions compared to the statutory petroleum baseline by 69%. This allows for the generation of advanced biofuel, or D-Code 5, RINs.

Table A-9. Permitting summary.

Agency / Permit	Discussion
Alcohol and Tobacco Tax and Trade Bureau (TTB) – Alcohol Fuel Producer Permit Level: Federal	A bureau of the Department of Treasury, TTB is primarily concerned with collecting excise taxes related to the production of alcohol. Algenol qualified as a small producer and obtained the corresponding permit.
Florida Department of Agriculture and Consumer Services – Aquaculture License Level: State	The biorefinery operates as a licensed zero-discharge aquaculture facility. The license is renewed annually and the facility is inspected twice per year and Algenol's record in this regard is incident free.
Florida Department of Environmental Protection – Class I Industrial Well	The wells constructed for the project were permitted as a class I industrial well and include both recovery and

Agency / Permit	Discussion
Approval Level: State	injection capabilities. All construction, maintenance and inspection requirements have been fulfilled.
Florida Department of Environmental Protection / Southwest Florida Water Management District - Environmental Resource Permit Level: State	The existing Environmental Resource Permit for the site regulating surface water flows, stormwater management and any impact to wetlands (no impact) was amended to allow for the construction and operation of the biorefinery.
Florida Department of Environmental Protection - Air Permit (Construction and Operation) Level: State (State of Florida is responsible for administering federal Clean Air Act permitting requirements)	The biorefinery was formally exempted from air permitting requirements because it is below the allowable threshold for any regulated emissions.
Lee County Building and Permitting Department Level: Local	Approve the project, the construction permits, conduct in-process construction inspections, issue final Certificate of Occupancy

Subtask A.8 Life Cycle Analysis update

Completed August 2010

See section B.5 Techno-Economic Analysis.

Subtask A.9 Program Management of DOE phase I activities

Completed August 2011

The Program Management Team controlled the budget, schedule and work within the project and managed the relationship between Algenol's collaborators at the Georgia Institute of Technology (Georgia Tech), Membrane Technology & Research Inc. (MTR), the National Renewable Energy Laboratory (NREL), and Dow.

A Work Breakdown Structure (WBS) was developed for all work required for the successful completion of the project. Each WBS element defined each subsystem's approved scope, cost, schedule, and the necessary resources to provide its commitments, and was the basis for all planning strategies and cost/schedule performance measurements. The WBS integration of planning, scheduling and budgeting of authorized work provided the basis for the Performance Management Baseline (PMB). The Project Manager and Project Accounting Manager (PAM) were responsible for maintaining the PMB.

The project Earned Value Management System (EVMS) was developed and was utilized throughout the life of the project. The EVMS used the WBS breakdown of the work scope into discreet elements for authorization of work and budget allocation. Each month, as work was completed, the Budgeted Cost of Work Scheduled (BCWS) was compared to the Budgeted Cost of Work Performed (BCWP) and a Schedule Variance (SV) was calculated. The BCWP was compared to Actual Cost of Work Performed (ACWP) to quantify the cost variance (CV). The earned value performance was submitted to and reviewed by Project Team as part of their standard monthly progress meetings. The PM, working with the individual project team technical

leads, was required to provide reasons for variances, either SV or CV, above 5% of the budgeted amount or $100,000, whichever is greater.

After reviewing the status of their earned value performance and budget/actual versus work accomplished, the project team technical leads took the necessary corrective actions (e.g., adding resources; rescheduling or de-scoping work; increasing budgets by requesting contingency) to keep on a realistic budget and scheduling path. Any recommended changes to the project budget by the project team technical leads were presented to the PM and vetted by the PMT. Once approved, the PAM incorporated the changes into the EVMS and all WBS's, estimated costs, schedules, scope, budgets estimate to complete and other relevant documents accordingly. The PAM conducted reconciliation of the sum of the internal program budgets to the PMB and overall project budget and reported to the PMT and DOE as required.

The main tool in measuring cost and schedule progress and variances and for tracking actual costs within each phase was the Cost Performance Report (CPR). The CPR followed the subsystem's WBS and was "rolled-up" to assess the performance for an individual task/element or for major portions of a project or subsystem.

A Risk Management Plan (RMP) was developed for the program that defined the scope and process for the identification, assessment, and management of risks that could impact the implementation of the program. The RMP included assessable risks that could potentially jeopardize successful completion of the project's technical, financial, and environmental goals. The plan defined the strategy to manage program-related risks throughout the project life cycle such that there was acceptable minimal impact on cost and schedule, as well as operational performance. The RMP was an evergreen document that was updated as needed to ensure that previously identified risks are managed effectively and new risks were quickly identified and managed throughout the life cycle. A Failure Mode Effects Analysis (FMEA) and other tools were used to identify the effects of uncertainty on costs and time. Variables considered include regulatory and stakeholder requirements, cost escalation, financial constraints, technology, resource availability, laboratory capacity and unexpected field conditions.

The PMT was responsible for effectively managing a given risk and updating the risk register. All identified risks were handled based on PMT recommendations (Figure A-50). Responses to various risks fell into four categories: reduce or mitigate, accept, avoid, or transfer. Risk priority and resource availability determined the execution sequence of each risk mitigation action. Project team technical leads, the project manager, subject matter experts, as appropriate, and key project staff members were responsible for ensuring that programmatic and project risks were identified, analyzed, mitigated, tracked, and trended. Project team members were responsible for execution of risk mitigation strategies and techniques.

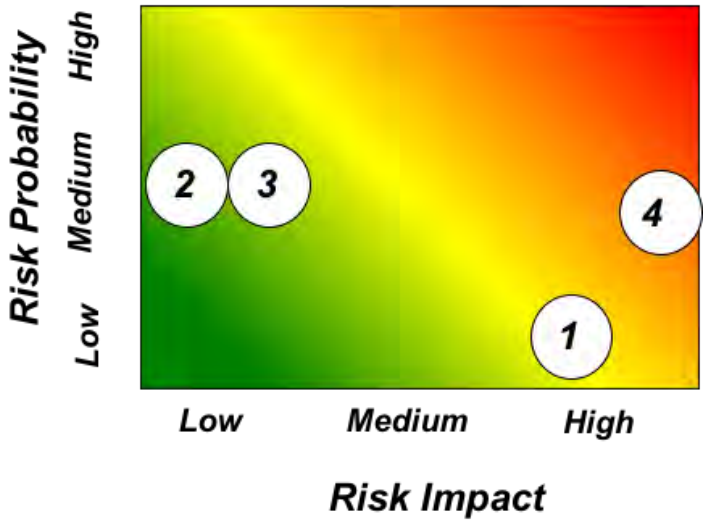


Figure A-50. Risk assessment diagram template used in IBR project management.

Subtask A.10 Phase I gate review

Completed August 2010

The Phase I gate criteria were proposed by the Project Management Team (PMT) at the initiation of the project, approved by the Internal Gatekeeper Team (IGT), and communicated to DOE. At the completion of Phase I, the internally defined Gate Criteria for Phase I required: 1) algae that make ethanol at a rate of more than 4 mmol per liter per week and are able to grow outdoors in photobioreactors containing salt water; 2) photobioreactors that produce ethanol/water mixture concentrations of 0.5% or more; 3) ethanol/water separation equipment that can process the condensate removed from the photobioreactor and produce an ~10% ethanol-water mixture suitable for conventional distillation; 4) satisfactory completion of NEPA review and regulatory approvals and permits; and 5) a life-cycle analysis that indicates at least a 60% reduction in greenhouse gas emissions.

Subtask A.11 Pre-award look back

Completed January 2010

In this subtask Algenol and the DOE reviewed award related R&D and determined which R&D could be reimbursed under the award.

Subtask A.12 On-going demonstration of organism performance at 4500L scale in salt water outdoors

Completed February 2011

Lab scale

Cultivation research along the path towards commercial demonstration of Algenol's ethanol production process first focused on evaluations of early hybrid strain growth and ethanologenesis in salt water medium and across scales. At the smallest scales, controlled condition laboratory experiments in 0.5 L culture volume Crison photobioreactors were performed to determine baseline productivity rates under ideal conditions and help in strain selection. First generation hybrid strains (constructs in *Synechocytis sp.*PCC 6803) showed good rates of ethanol production, even as the culture progressed into stationary phase, with volumetric production rates of 0.027% v/v per day and 0.030 % v/v per day for #309 and #550,

respectively (Figure A-51). Later strains, based on ABCC1535 and AB1, were also pursued for improved temperature tolerance, with AB0015 (AB1:TK293) showing favorable ethanol production rates on the order of those found for ethanologenic PCC 6803 derivatives (Figure A-52). Candidate hybrid strains were then progressed up to laboratory 10 L cultures for large-scale indoor trials, with successful strains meeting and typically exceeding the Phase I ethanol production rate target of 2000 gallons of ethanol per acre per year (GEPAY).

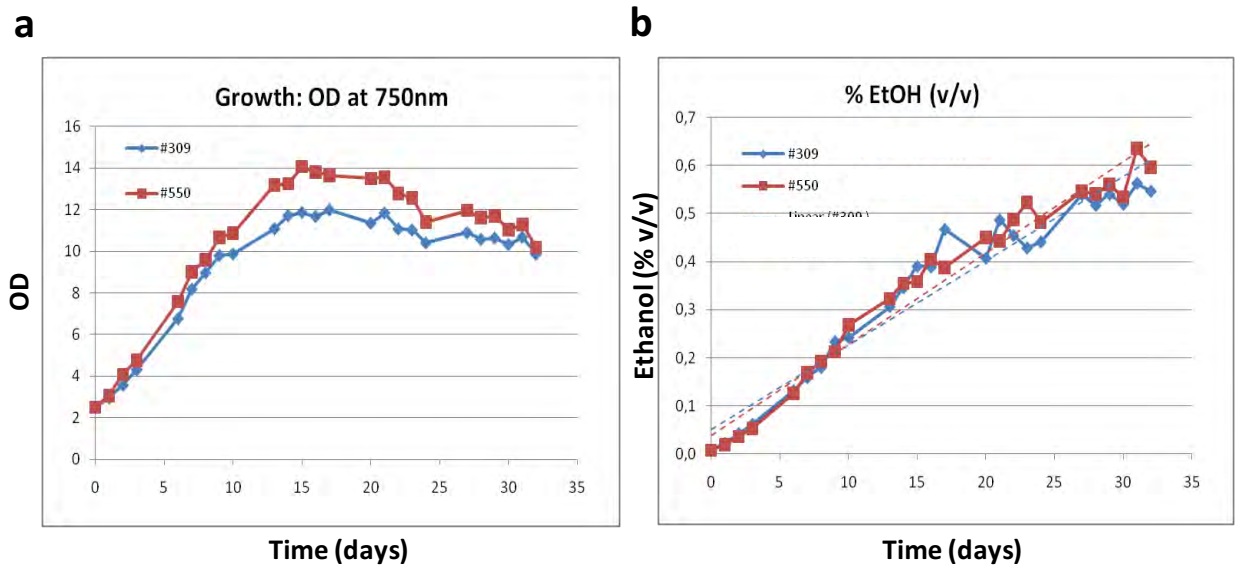


Figure A-51. Growth (a) and ethanol production (b) in two hybrids of PCC 6803, with volumetric production rates of 0.027 %v/v per day for #309 and 0.030% v/v per day for #550.

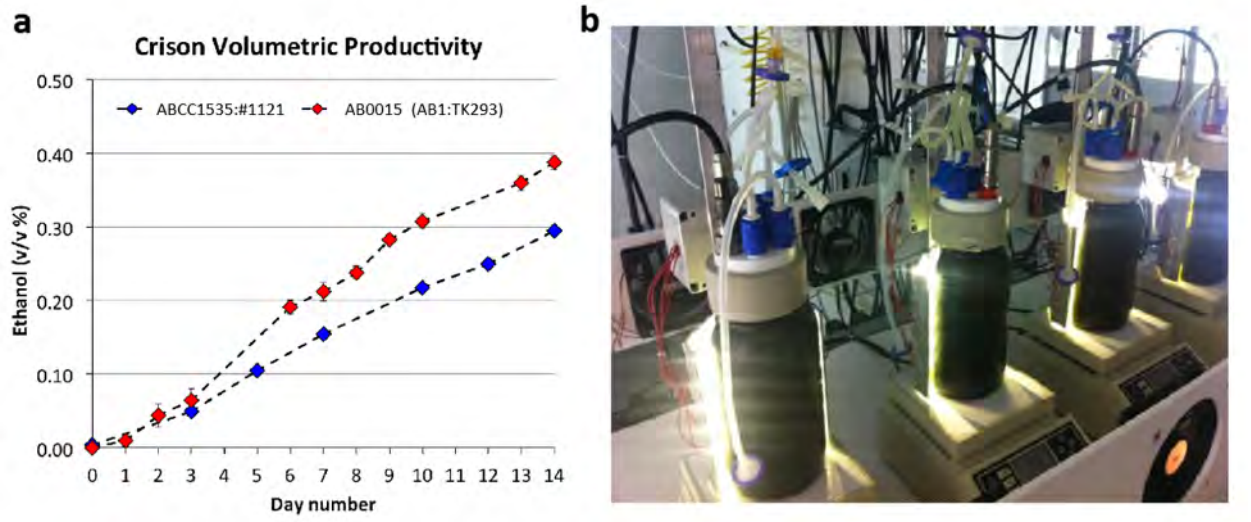


Figure A-52. a). Lab-scale ethanol production in two hybrid strains showing productivity of 0.021 %v/v per day for ABCC1535:#1121 and 0.030 % v/v per day for AB0015 cultivated in marine medium. b). Photograph of laboratory Crison experimental set-up (0.5 L culture volume).

Lab-scale cultivations were very important for rapid advancement of the technology because of their relative ease of set-up, ability for high levels of replication, highly-controlled conditions, and small volume inoculum and medium demands. Because of these benefits, small-scale laboratory experiments continued concurrently with outdoor scale progression, largely with the

intents of determining potential limiting factors (dissolved organics, oxygen, nutrients, etc.) for ethanol production, evaluating new strains, and testing pathways for mitigating emergent outdoor cultivation challenges. Thus, progression from small to large scales was not strictly linear, but instead advanced circuitously with combined efforts from indoor and outdoor cultivation.

Outdoor process development scale

Building on laboratory strain evaluation successes and culture management guidance, outdoor cultivation trials began first at the FARM site in Loxahatchee, FL and subsequently at the Process Development Unit (PDU) at Algenol's consolidated Fort Myers, FL location. These initial outdoor experiments used both wild type and early hybrid strains, focusing on medium preparation, CO_2 addition, sterilization, and inoculation procedures. Outdoor production test systems at this time included ~50 L GE horizontal rocker bags, 60 L and 150 L foil-mixed horizontal photobioreactors (PBRs), high volume (500-1000 L horizontal) research PBRs (rPBR), and commercial scale industrial (4500 L) PBRs (iPBR; Figure A-53).

25-50 L Rocker Bag | **150 L research photobioreactor (rPBR)** | **500-1000 L research photobioreactor (rPBR)** | **4500 L industrial scale photobioreactor (iPBR)**

Figure A-53. Examples of outdoor horizontal photobioreactor systems in use during Phase I.

The first growth and ethanol production evaluation at the 50 L scale was conducted in GE rocker bag PBRs during April 2010 in the PDU greenhouse with a 1st generation hybrid strain (hybrid PCC 6803), with the objective of evaluating strain performance (Figure A-54a). Replicate (n = 5) bags produced ethanol for 24-45 days. Averaged rates of ethanol accumulation over the initial period of production met the Phase 1 gate criterion of 2000 GEPAY for 30-60 days at 50 L (Figure A-54a). Progression through larger scales continued over the summer and autumn of 2010 to 500 L culture volumes. Initial production rates at this scale also exceeded 2000 GEPAY; however, rates began to drop as temperature and light levels decreased in autumn (Figure A-54b).

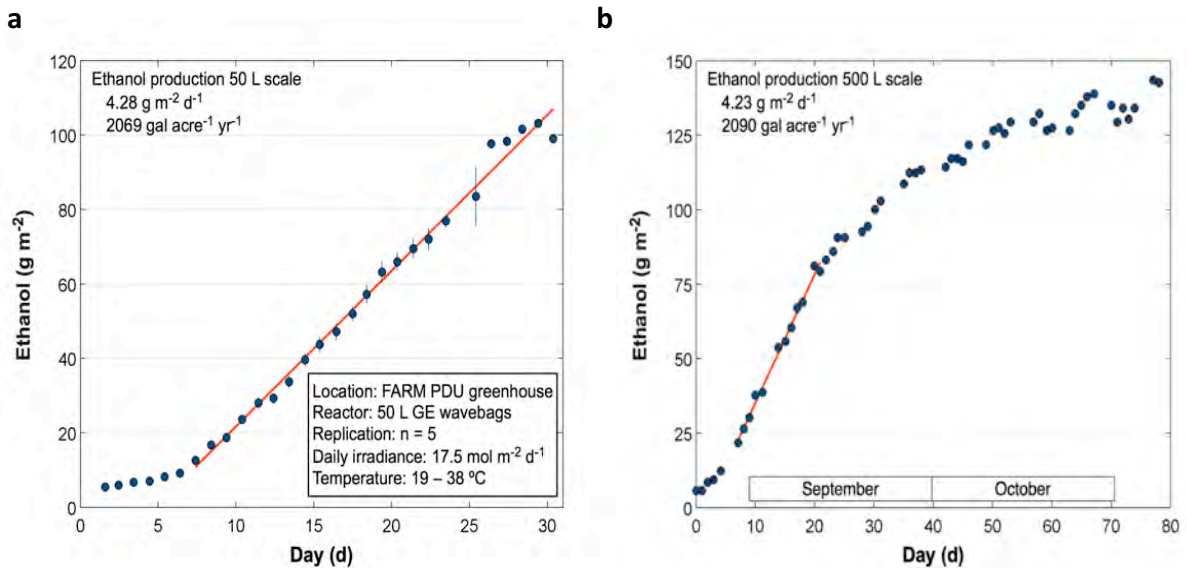


Figure A-54. Ethanol accumulation at the 50 L (a) and 500 L (b) scale in the first large scale outdoor experiments conducted at the PDU during 2010. Ethanol production rates were calculated for the time period marked in red for each dataset with rates just above 2000 GEPAY.

For the next step along demonstrated growth at larger scales, two integrated PBRs configured with a prototype mixing system and an ethanol collection system were built at approximately one-fifth of the planned size for the iPBR (925 L). These PBRs were contained in an indoor temperature and light controlled room designed to simulate many aspects of an outdoor environment. Preliminary experiments during December 2010 and January 2011 suffered from mixing system difficulties and contamination by bacteria and fungi; however, these challenges provided important contamination control data leading toward modifications of ozone PBR sterilization procedures and mixing system cleaning and mechanical improvements (see below).

Contamination control activities and accomplishments – Common cultivation challenges during this research period were high temperature (>45°C) intolerance of hybrid PCC 6803, rapid losses of ethanol typically associated with compromised PBR containment, and mixing system mechanical and sterility failures. To advance our knowledge on contamination control, a dedicated research team was established to examine contamination as a limiting process for ethanol production and develop strategies for control of heterotrophic bacteria and fungi for extended time periods in the photobioreactors. In addition, considerable research efforts were put forth to develop large-scale PBR sterilization methods. Hypersalinity (2X marine (salt water) salt levels) was one of the first contamination control strategies to be evaluated and showed that elevated salt concentrations could restrict the growth of contaminating species within the modified cyanobacterial cultures; however, these same salt conditions reduced the rate of biomass accumulation for the first-generation hybrid strain. As such, wildtype strains were evaluated for salt tolerance for introduction into the transformation program for the generation of ethanologenic organisms. In addition, collections of contaminating organisms, both fungal and bacterial, and defined by their ability to use ethanol as a carbon source, were made in Florida. These organisms were cultured to homogeneity, species identified, and used for contamination control strategy lab testing, particularly with respect to naturally-derived metabolites from strain ABCC1535 and commercially-available antibacterial and antifungal compounds as inhibitors of ethanol consumption.

PBR sterilization experiments were conducted in part to determine if more effective initial sterilization could improve the durations of ethanol productivity. These experiments highlighted that the mixing system was a vector for contamination and led to increased ozone concentration and lengthened ozone residence times in the PBR to extend the ethanol production window.

Specific achievements during Phase I

- Cultivated first generation hybrid strain (hybrid of PCC 6803) outdoors at scales up to 925 L and began testing of new strain ABCC1535
- Passed internal gate review by demonstrating production rates over 2000 GEPAY at the 50 and 500 L scale
- Implemented a basic cultivation medium (BG-11 nutrients in salt water) for rapid assessment of mineral nutritional status of culture by following rates of nitrate utilization
- Developed CO_2 demand control systems based on culture pH with real-time data acquisition software
- Designed, implemented, and improved foil mixing systems for horizontal photobioreactors with respect to mechanical integrity and cleanability
- Initiated Contamination Control and Duration of Productivity Project Teams
- Developed ozone sterilization protocols for large-scale (4500 L) photobioreactors and salt water

At the transition between Phases I and II, the production process consisted predominantly of outdoor cultivation of preliminary ethanologenic hybrid strains based on PCC 6803 and ABCC1535 in horizontal rocker (50 L scale) or foil-mixed (150 L scale) photobioreactors for research experiments. For smaller scale R&D experiments, gamma irradiation of bioreactors and autoclaving of media were used for general experiments not investigating sterilization methods; however, at larger scales and for targeted sterilization experiments, ozone was the sterilant of choice for both cultivation materials and salt water. Cultivation was in marine BG-11, often with vitamin and antifungal additions; however, medium optimization was in preliminary phases. Ethanol production rates for short duration experiments reached 5000 ± 2500 at small scales, 2500 ± 1000 at the 50 L scale, and 1500 ± 200 at the 60-150 L scale. Although rates for selected experiments met or surpassed the Phase I gate criteria, overall the results indicated a need for improvement, particularly for the next project phases. Technological challenges leading to lower than desired ethanol production rates going in to Phase II were as follows:

- Low tolerance of hybrid strains in horizontal bioreactors to high temperature and high light typical of Florida
- Intolerance of early hybrid strains to high oxygen concentrations
- Frequent losses of ethanol due to consumption
- Short durations of maximum productivity
- Challenges with mechanical mixing systems in horizontal bioreactors
- Non-optimized growth medium

Cultivation research in Phases II and III addressed these challenges and led to major advancements of commercial ethanol production technology (see Task B.1 summary).

Subtask A.13 Initiate architect/EPC firm associated fees: moving forward with an EPC firm

Completed February 2011

After the review process (Table A-10), Algenol chose Applied Engineering and Management (AEM) Corporation to act as the EPC for the water systems of the plant. Dave Binning, Director, Infrastructure Programs at AEM was the project manager. Dave is a water and wastewater engineer and has over 40 years experience working on large and small water and wastewater systems around the world.

Algenol chose Richard Ditter Architecture as the local project architect. Richard Ditter was the primary architect on a previous $10 million Algenol project in Fort Myers. Since Florida has very unique building codes, working with a local architect ensured that all local code requirements were met.

Quattrone & Associates, Inc. was chosen as the site civil engineering firm. Quattrone & Associates worked on a prior project at the Fort Myers site and had detailed knowledge of the area and property of the project. Quattrone completed the preliminary and final site development plans, the paving, grading and drainage plan, utilities plan and permitting, traffic analysis, landscape plan, site development permitting and the Environmental Resources Permit Modification with the South Florida Water Management District.

Cardno ENTRIX was chosen as the contractor for the Class I injection/supply well design, permitting, construction and testing. Cardno ENTRIX has extensive experience completing supply and injection well projects in south Florida.

GCM Contracting Solutions was chosen as the project general contractor. GCM was the general contractor on a previous $10 million Algenol project in Fort Myers.

Southeast Drilling Services was chosen as the well drilling contractor.

Linde Process Plants was chosen to conduct final design and develop shop drawings for the construction of the Vapor Compression Steam Stripper.

Table A-10. Final design specifications.

Design Function	Date Completed	Notes
Pilot Plant Design	August 2011	AEM completed the overall IBR design
ASR Well Design	July 2011	Cardno Entrix completed the well package and submitted it to FDEP and released for bid
Civil Land Design	April 2011	Quattrone completed the civil package and submitted it for permitting review
Buildings Design	May 2011	Richard Ditter completed all design documents for submittal for permitting review
Pre-fab steel buildings	April 2011	Olympia Steel Buildings completed all building design documents for submittal for permitting review
Ethanol Processing Area Design	July 2011	Algenol and Linde completed the downstream processing package to be released for bid
Flexible Film PBR Block Design	July 2011	Algenol completed the block design to be incorporated into plant designs
Control System Design	July 2011	Algenol completed the plant control logic and submitted to Honeywell for controls systems

Design Function	Date Completed	Notes
		design

Subtask A.14 Compile final design specifications

Completed August 2011

Preliminary site lay-outs and scaled drawings were developed for the Oyster Creek and Fort Myers sites. Detailed preliminary site specifications were delivered in August 2010 from Algenol and AEM Corporation for design work and provided to RW Beck for inclusion into the EIR-2 Report.

In 2011, the various teams completed final design specifications for all equipment and systems planned for the biorefinery, which included any recommendations as a result of the NEPA determination and all research and development from Phase I activities. These specifications were turned over to AEM Corporation, Richard Ditter and Cardno ENTRIX for completions of final design documents for submittal to appropriate regulatory agencies. Submittals to Lee County for permitting did not include equipment designs for the processing area.

Subtask A.15 On-going recruitment of Plant Manager, Engineer, and Project Manager

Completed August 2011

Algenol was able to assign certain of its existing employees to the IBR. Most of the personnel working in the biology function of the IBR were transferred from Algenol's research and development department to the IBR. All other personnel were hired from the local talent pool in southwest Florida. Hiring qualified individuals was not difficult as the local economy was in a severe down-turn. The plant manager and plant engineer were assigned from Algenol's existing personnel. The plant construction manager position was filled by Ed Legere, the project co-PI at the time.

Subtask A.16 Continued flexible film photobioreactor evaluation

Completed August 2011

Refer to Task A.3 for all PBR data.

Subtask A.17 Program management of extended DOE Phase I activities

Completed August 2011

This is an extension of A.9 using the same processes and structure.

Subtask A.18 Extension 2

Completed August 2011

This is an extension to allow more research and development time before passing through the gate and into Task B.

Task B – Build a Pilot Scale Biorefinery

Task Objective

In Task B Algenol signed a construction contract with a general contractor and built the Pilot Plant according to the final construction blueprints prepared by the architect and approved by

the local regulatory authorities. Algenol personnel were assigned to the IBR to operate the facility. During the construction of the IBR, these personnel were trained to run the facility at Algenol's PDU in Florida. In addition, IBR and PDU personnel transferred and wrote Standard Operating Procedures (SOPs) for the IBR based on the SOPs developed and used at the PDU. Upon completion of the construction of the IBR, system checks and shake down runs were performed. Following these activities, an independent engineer evaluated and verified the process and issued a report.

Project Activities

Subtask B.1 Continued demonstration of organism performance at scale

Completed October 2014

Research during the early part of this budget period focused on the causal factors for sub-optimal ethanol production including a review of PBR design and function. Modifications to PBR design proposed for evaluation at that time included vertical orientation with air lift technology for mixing, reduced volume horizontal PBR configurations, and modified mixing systems to improve gas phase/liquid phase exchange. Experiments began to assess the roles of various PBR-derived parameters such as light dilution, dissolved oxygen concentrations, inorganic carbon availability, and temperature regulation as modified by PBR configuration.

As a result of this focus, Phase II (2012-2014) was a time of tremendous progress toward commercial demonstration. Personnel gains in multiple departments allowed for expanded experimental capacity. Experiments on the relationship between PBR design/operation and productivity concurrent with strain improvements proved pivotal for Algenol in 2012. Foremost, a series of outdoor experiments evaluated several new PBR designs out of the PBR Project Team including hybrid horizontal/vertical PBRs and new vertically-deployed PBRs and compared them with standard horizontal research PBRs. These experiments demonstrated huge improvements in performance in vertically-oriented growth systems compared to horizontal platforms (Figure B-1 and Figure B-2). The vertical PBRs were found to solve four main challenges identified from horizontal designs:

1. Better light utilization—more illuminated culture surface area per PBR footprint area and mitigation of over-saturating light conditions
2. Lower temperature—maximum daily temperatures in the summer are approximately 10°C less in vertical *vs* horizontal systems (Figure B-3a)
3. Oxygen management—bubbling promotes gas exchange and culture dissolved O_2 concentrations are generally <200% saturation (Figure B-3b)
4. Population stability—preliminary data suggested that with lower temperature, oxygen concentrations, and/or light stress, cells producing ethanol at high levels could persist longer in the culture.

In addition, vertical PBRs afforded economic gains from their ability to be inoculated at a lower cell density, use lower water volumes for a given production area, and enable higher ethanol productivity compared to horizontal PBRs. Based on all of these advantages, experiments and commercial planning switched to the vertical PBR platform.

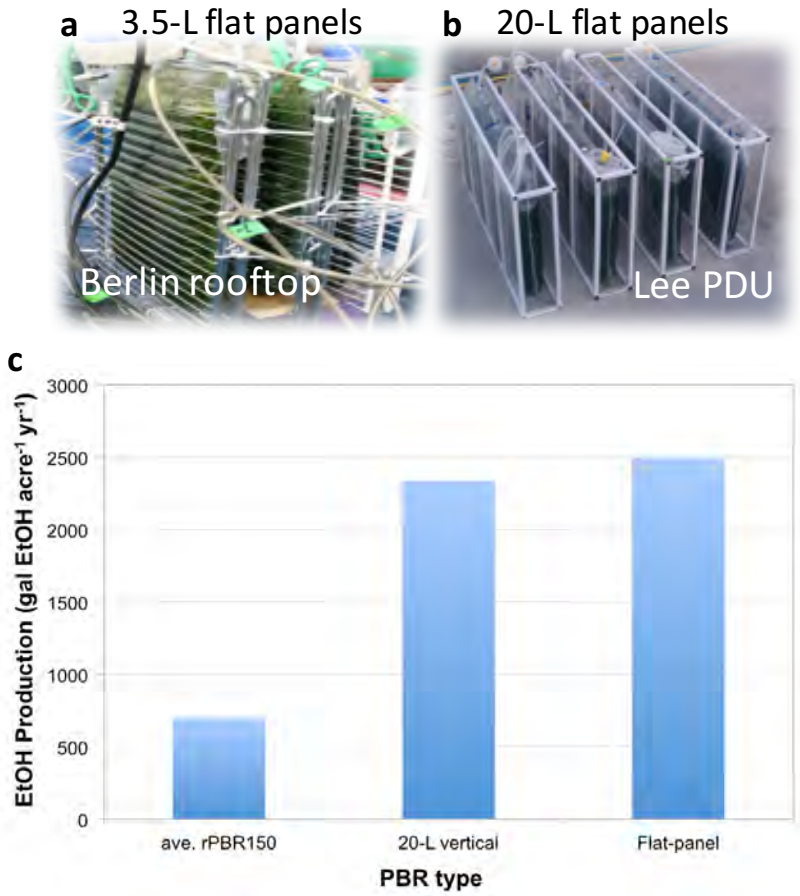


Figure B-1. Examples of early vertical photobioreactor prototypes (a, b) and preliminary data comparing horizontal (rPBR150) PBR ethanol productivity to prototype non-optimized vertical column and flat-panel PBRs (c) using the same ethanologenic strain.

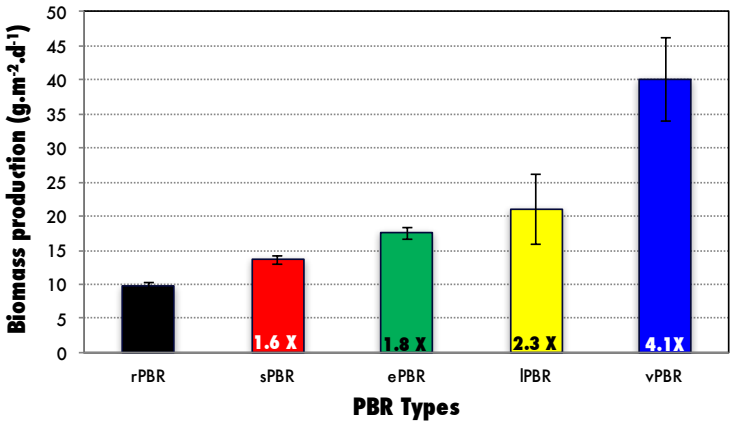


Figure B-2. Improved productivity of wild type strain in all new photobioreactor types compared to original (rPBR) design. The largest improvement was demonstrated in the vertical platform. rPBR = research 150 L horizontal PBR; sPBR = skimboard mixing rPBR; ePBR = enclosed light panel rPBR; lPBR = rPBR with light pipes for improved light distribution; vPBR = vertically oriented PBR.

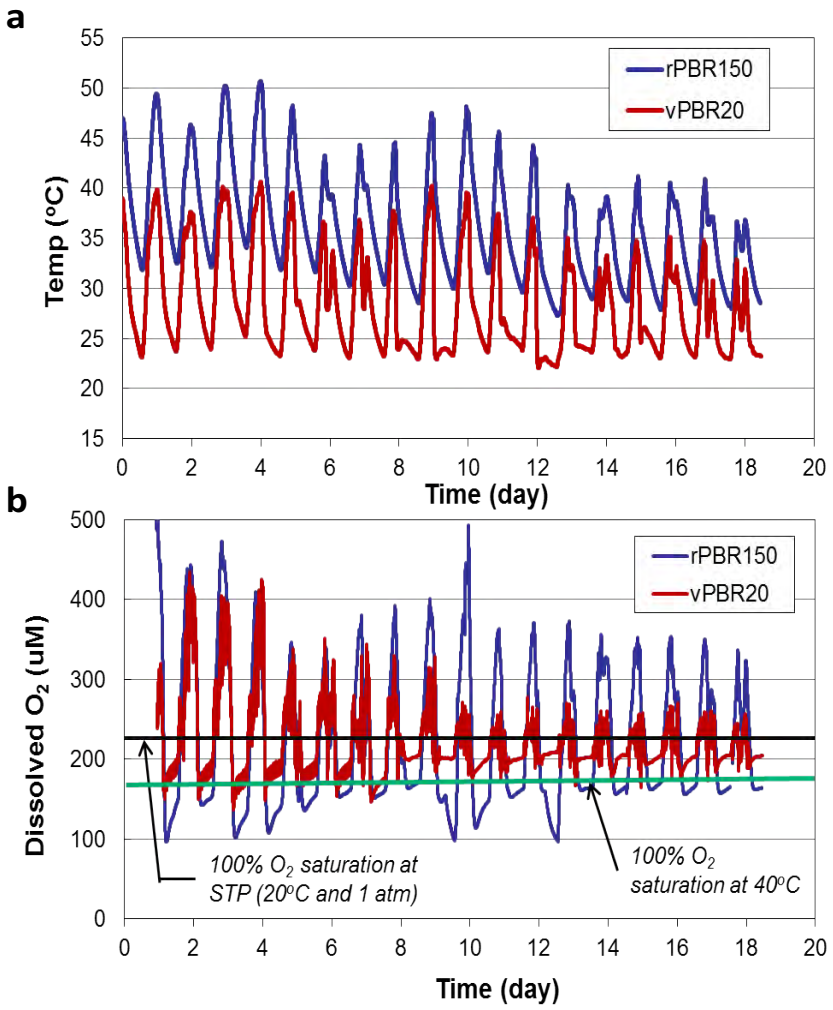


Figure B-3. Comparison of culture temperature (a) and dissolved oxygen concentration (b) in horizontal (rPBR150) versus vertical (vPBR20) PBR systems.

Following the switch to vertical photobioreactors, several interdependent project teams were established to make rapid progress with respect to ethanol production rates that could be translated to commercial scales. Under these teams, lab-scale and PDU scale experiments focused on advancing our knowledge of strain characterization and development, culture optimization, contamination control, inoculum production, and field cultivation (including nutrient management, culture operations, and gas management), with the intent of rapidly transferring new technologies to the IBR.

Lab-scale achievements toward commercialization during Phase II

A lab scale version of the vertical PBR (LvPBR; Figure B-4a) was developed to mimic a single channel of the outdoor vertical photobioreactor with similar results observed for 2nd generation hybrid strains between indoor and outdoor experiments (Figure B-4b, c). This newly validated lab platform allowed for large, complex studies evaluating interactions between cultivation parameters such as temperature, irradiance, oxygen, inorganic carbon, and mixing; culture management concepts such as batch mode *versus* dilution and impacts on productivity and strain genetic stability; and nutrient optimization. As progress was made at this scale, the knowledge was transferred to larger and larger scales outdoors for process validation.

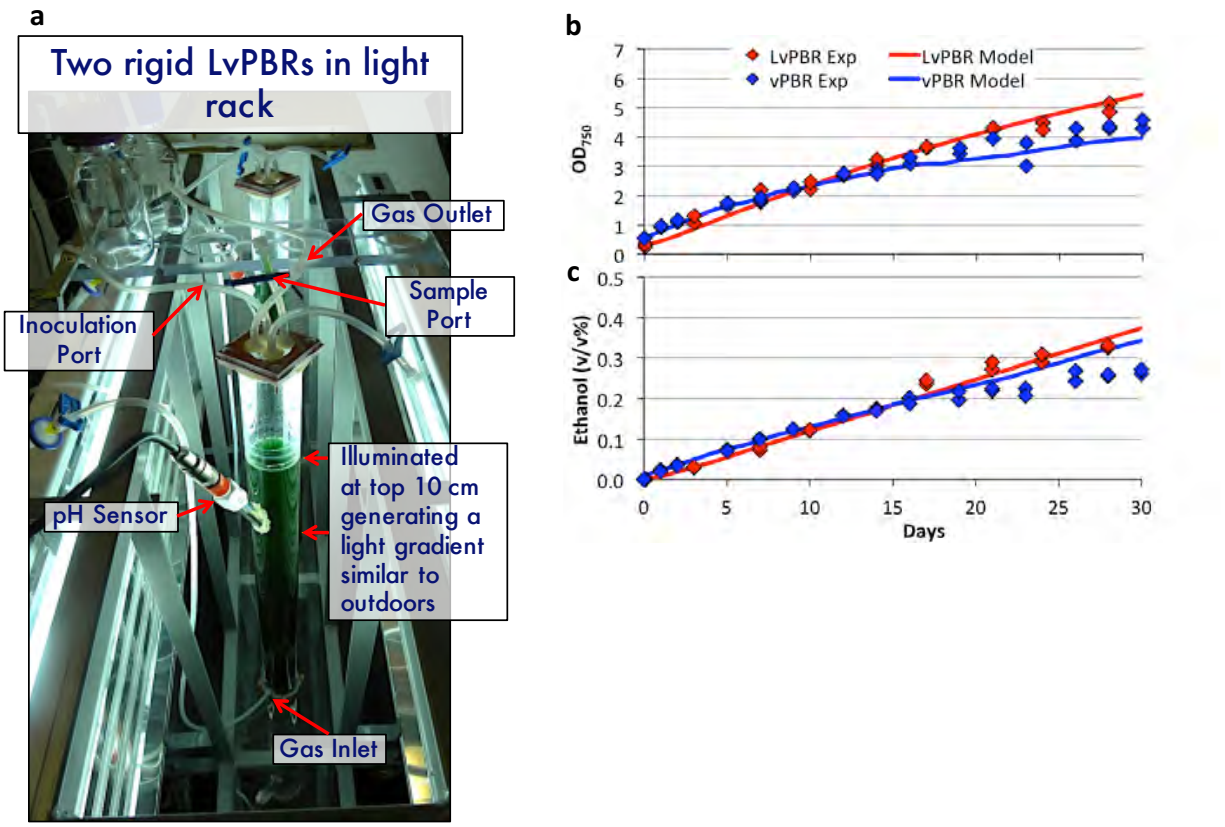


Figure B-4. Photograph of two lab-scale vertical photobioreactors (LvPBR) designed to be analogous to a single column of the outdoor vPBR prototype. Biomass (b) and ethanol concentration (c) comparisons between LvPBRs grown at 170 µmol photons/m^2/s two-sided illumination and vPBRs deployed in the Limits 1 outdoor experiment.

Nutrient optimization – Optimal medium composition and feeding strategies were widely explored in the LvPBR platform. Nitrate utilization experiments were performed to determine culture N-demand under batch addition and daily feeding scenarios.

Major achievements from indoor cultivation during this time period:

- Performed large experiment investigating effects and interactions of oxygen level, total light, mixing rate, peak light, dissolved inorganic carbon (DIC) content and temperature which highlighted the need for low O_2 concentrations, high mixing rate, and intermediate levels of DIC for maximum ethanol productivity
- Empirically determined nitrogen demand and phosphorus growth and storage needs, and passed recommendations for nutrient feeding protocols to larger outdoor scales
- Validated suitability of Algenol's new saline water well as a cultivation medium

PDU-scale research during Phase II

At the same time, multiple lines of research outside sought to validate lab results and optimize vertical PBR operation by focusing on PBR spacing and orientation, culture management (operational paradigm and nutrient optimization), and gas management.

Several experiments were conducted to investigate the impact of PBR spacing and orientation on light availability and light use efficiency. Results of an initial experiment during the limits program, which showed that widening the distance between vertical panel faces increased ethanol concentration without substantially changing areal productivity, led to a full spacing experiment to test a wide variety of panel interface distances. Optimizing the spacing led to increases in productivity and ethanol concentration while concurrently decreasing the cost of separations, support structures, aeration, and PBRs.

Research in gas management focused on CO_2 delivery and O_2 management both for gas recycle and venting states. Gas recycle experiments determined oxygen tolerance thresholds for commercial gas recycling and demonstrated that similar ethanol production rates could be achieved outdoors between venting and recycling states.

Additional major R&D achievements outdoors during Phase II included the following:

- Demonstrated process integration with operation of connected rows of photobioreactors with experiments focusing on batch operation separated by CIP compared to dilution
- Validated outdoors optimal nitrogen and phosphorus feeding strategies developed in LvPBRs
- Evaluated proposed commercial strain and commercial diffuser/reactor type and made recommendations for industrial scale (IBR) use
- Demonstrated >5000 GEPAY at the row scale for 30 days

Scale-progression and major accomplishments for IBR cultivation activities during Phase II

The first IBR cultivations began at this time based on the recommendations delivered from the aforementioned lab and PDU experiments and progressed in scale from blocks of 40 to 400 to a 4000-module consisting of 5 blocks of 800 vPBRs (Figure B-5).

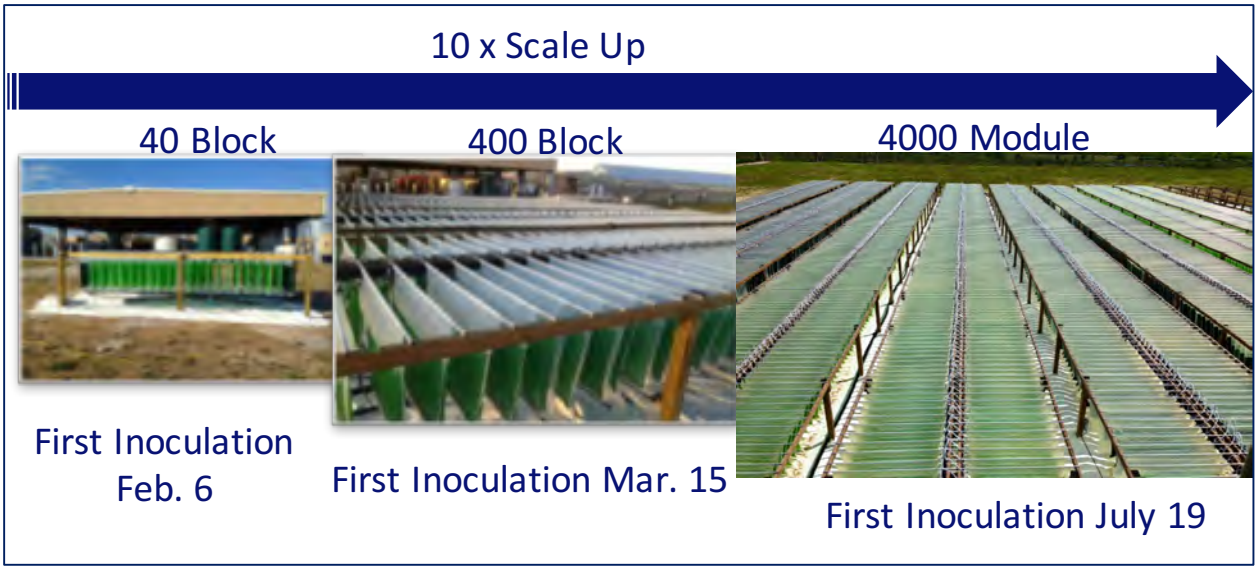


Figure B-5. Photographs of the 40 and 400 Blocks and 4000 Module showing the scale progression and first inoculation dates (2013) for cultivations in VIPER1 photobioreactors at the IBR during 2013-2014.

40-Block – 40-block cultivations began with integrated block ozone sterilization demonstrations, with sequential batches building on previous successes with the additions of CO_2 control, initial sterilization modifications, CIP, dilution, and nutrient management strategies while evaluating long-term maintenance of ethanol productivity. The first run evaluated sterilization of a block of 40 integrated vPBRs and associated salt water with an ozone procedure. Contamination control (CC) medium, which is enriched in nutrients that support bacterial growth, was added to the sterilized well water to promote the growth of any residual contaminants. Although samples of the CC medium were free of contaminants for 14 days, contamination was evident on day 15 in half (20) of the vPBRs. Because the CC medium test is an exceptionally stringent test for sterilization, and the previous test was contamination-free for 14 days, the second run progressed to evaluate inoculation of hybrid ethanogenic AB1 in a block of 40 integrated vPBRs sterilized by the ozone procedure. The vPBRs were inoculated on Dec 4, 2012 and ran for >30 days with no detectable contaminants and no ethanol decline observed by day 45 when the platform was turned over to the contamination control team for targeted experiments.

The third 40-block run again used ozone for PBR and salt water sterilization for the 40-block, with inoculation in early February 2013 and a target of 30 days of ethanol productivity. Despite the presence of contaminants in almost all of the photobioreactors, ethanol was still accumulating, thus meeting the experimental target. Ethanol continued to accumulate until day 51, although with declining rates (Figure B-6). A dilution test was performed (76% dilution) with the goal of understanding if ethanol productivity would return to initial batch rates post-dilution. Although the rate did not recover all the way to initial batch rates, it was close and was substantially higher than rates just prior to dilution, indicating that culture management using several dilutions may be a viable method for extending ethanol productivity. The primary purpose of this cultivation was to further test dilution culture management strategies. Three dilutions were performed for a total of four batches post-CIP. Ethanol accumulation rates were found to decrease after each sequential dilution (Figure B-6).

The final deployment of the 40-block (#4) ran for approximately six months with the initial objective of finalizing culture management operations with respect to dilution and CIP timing between batches and a goal of achieving 180 days of production at 6000 GEPAY. Following a decline of rates following the first dilution, the decision was made to progress in batch mode with every batch separated by a CIP. The block ran for more than 180 days with all but two of the batches exceeding 5000 GEPAY and 6000 total gallons of liquid fuel (TGOLF; Figure B-7).

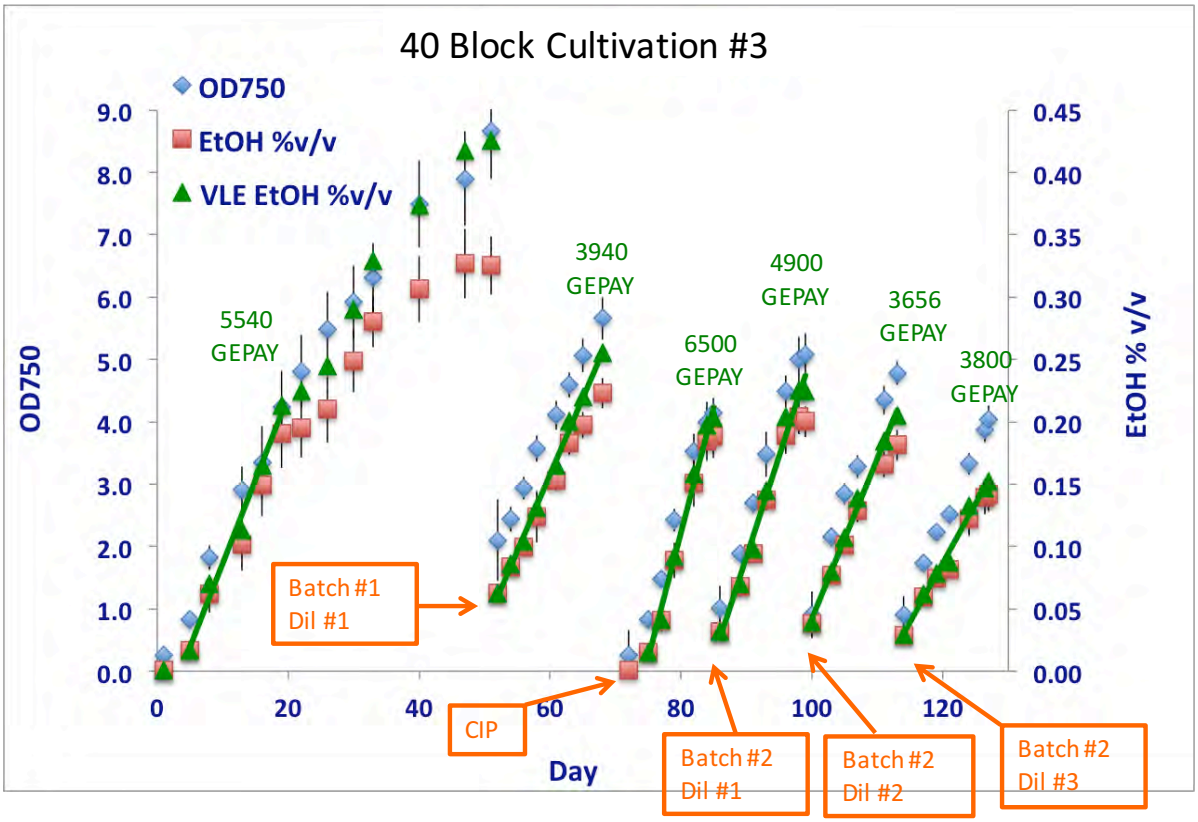


Figure B-6. Biomass as OD_{750} (left axis) and VLE-corrected ethanol concentration (right axis) for cultivation of AB1 hybrid strains at the IBR 40-block set #3 showing ethanol production rates following multiple batches separated by CIP or dilution.

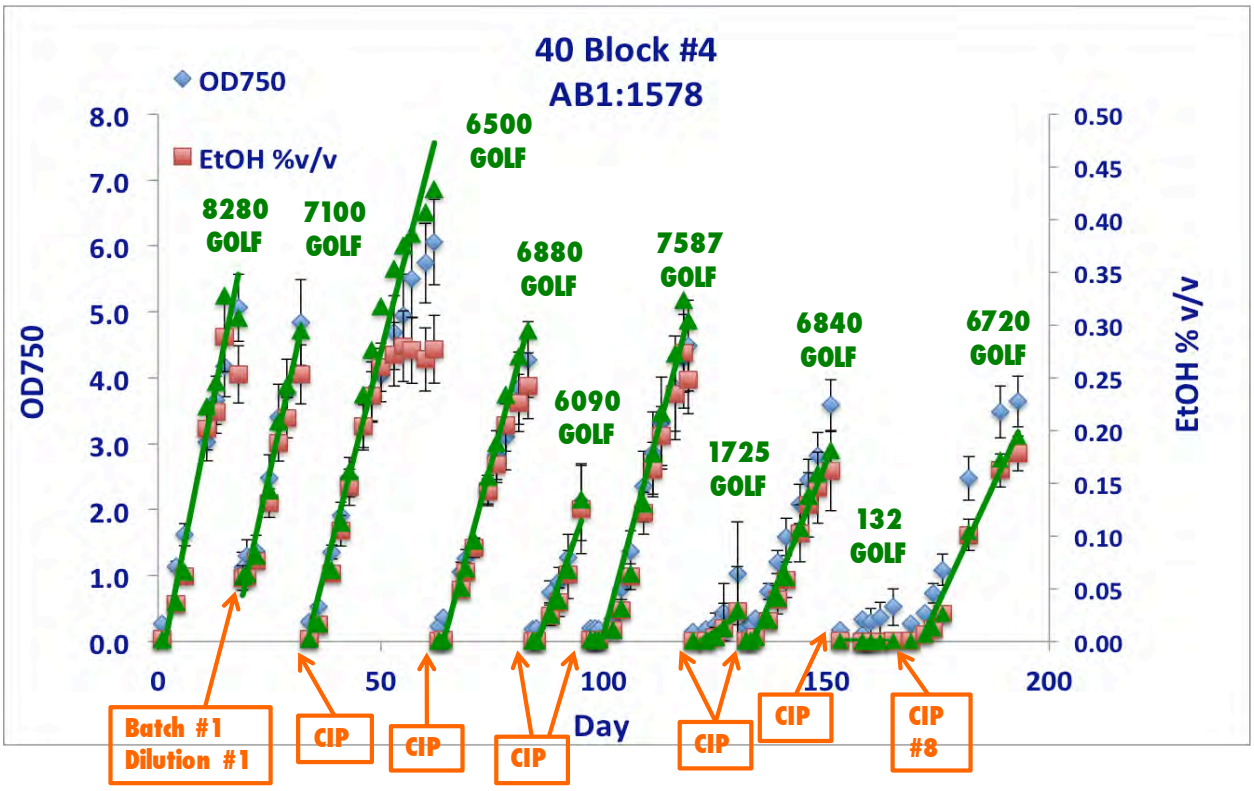


Figure B-7. Biomass as OD750 (left axis) and VLE-corrected ethanol concentration (right axis) for cultivation of AB1 hybrid strains at the IBR 40-block set #4 showing ethanol production rates following multiple batches separated by CIP or dilution.

400-Block – The next scale targeted sterilizing and inoculating a fully integrated block of 400 vPBRs with a 30-day batch followed by a CIP and a second batch. This experiment represented a leap to commercial demonstration at a 0.01.8-acre scale and required inoculum production for a large-scale cultivation. The initial batch was run for 38 days with continuous ethanol production despite the presence of contaminants.

4000-Module – The 4000-module represented the next leap in scale, with five blocks of 800 vPBRs as operational units. The five blocks comprising the module were operated between July 2013 and December 2014. The large number of batches run and the increasing complexity with larger scale in the five blocks provided an excellent opportunity for learning and making progress. While there were definite challenges with PBR and other material failures, operational errors, and contamination, Blocks B, C, D, and E had multiple sequential batches that demonstrated batch-to-batch consistency of production. Furthermore, these blocks also showed improvements in 2014 ethanol production rates for most batches over 2013 rates. The following other benefits were realized from this exercise in scale progression:

- Advancement in our knowledge of necessary ozone concentrations for cleaning/sterilization of previously used PBRs
- Development of CIP methods that incorporated liquid chemical soaking steps to improve PBR cleaning
- Manufacturing changes and implementation of in-house quality control testing to decrease PBR seam and component failures
- Improvement of SOPs and operational checklists to minimize human error

- Development of large-scale inoculum production methods which resulted in the axenic scale-up of >20,000 L in 2013 (Figure B-8)
- Transition from 6” light path scale-up PBRs for inoculum production to thin (1”) COBRA (**CO**mpressed **B**ioreactor for **R**epressed **A**lgae) PBRs to increase growth rate and improve light acclimation state of inoculum
- Testing and optimization of inoculum harvest density and production PBR inoculation density
- Development of SOPs for commercial scale sterilization of integrated PBRs and salt water
- Demonstration of recommended gas management and nutrient delivery strategies to large blocks of integrated PBRs
- Establishment of change control procedures through development and use of Algenol Recommended Cultivation Standards (ARCS) with Technical Committee approval for major operational changes

Figure B-8. Photograph of large-scale flat-panel inoculum production PBRs in the IBR greenhouse.

Based on the challenges of the 4000-module, the “100-block” program was initiated; this program was designed to incorporate advanced industry techniques for rapid process advancement in order to de-risk the 1.8-acre demonstration plant. These techniques included Failure Mode and Effects Analysis (FMEA) and Root Cause Analysis (RCA). The 149 failure modes identified in the FMEA led to a cultivation system redesign, which was implemented at the 100-block. At its inception in early 2014, the 100-block consisted of 4 independent rows of 26 10ft VIPER 2 PBRs which had already progressed through PDU evaluation (Figure B-9). The 100-block also had a newly designed steel frame support structure, new piping and tubing assemblies with more robust materials, and vacuum-assisted draining to minimize contaminant carryover between batches. Water-only testing was performed before initial inoculation to catch additional failure modes.

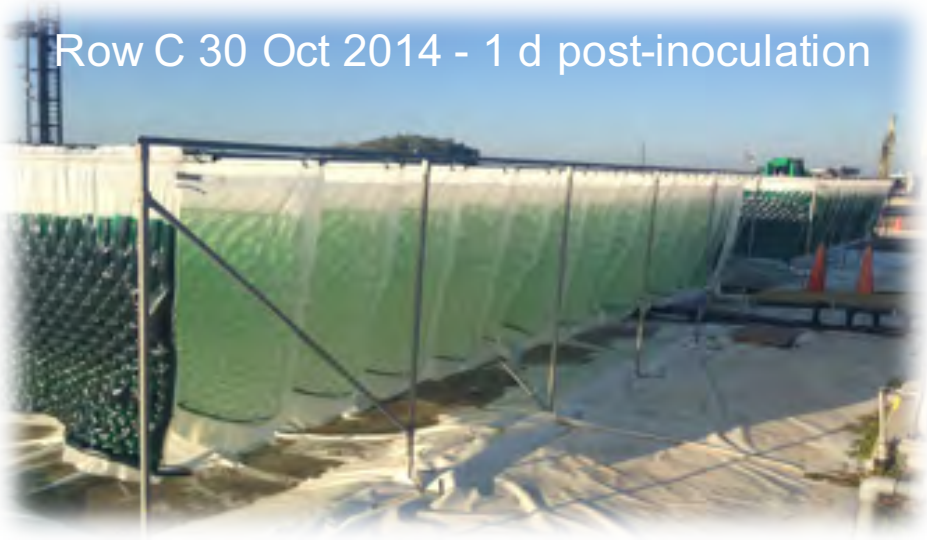


Figure B-9. Photograph of Row C of the 100-Block showing early phase cultivation of AB0005.

Nine batches were run in the 100-block rows between April and October 2014, which provided critical knowledge with respect to system design and performance for advancement to the 1.8-acre design team. As PBR design and cultivation management practices advanced at the PDU through 2014 and 2015, new cultivation platforms, process controls, and culture management techniques were brought to the 100-block for evaluation in a commercially relevant system. For example, in early 2015, the 100-block was modified to an airlift with 50 interconnected VIPER 3.2. The airlift design allowed for semi-continuous operation, simultaneous and even filling (or draining) of all PBRs, and improved culture and nutrient homogenization. Six cultivation batches were performed in 100-block airlifts before the first airlift inoculations at the 1.8-acre site in order to validate the design at a larger scale and develop operational checklists and cultivation practices for 1.8-acre deployments. Major achievements of the 100-block were as follows:

- Validated integrated VIPER 2.3 PBR and VIPER 3.x airlift system design
- Finalized 1.8-acre PBR, piping, and pad design features
- Validated robust CIP as a start-up disinfection method
- Demonstrated rapid (<22hr) CIP and re-inoculation process
- Demonstrated efficient CIP procedure relying solely on liquid contact of surfaces
- Validated co-flow procedure to fill and inoculate PBRs
- Delivered standard cultivation practices (ARCS), SOP templates, and operational checklists for 1.8-acre deployment

Contamination Control Activities and Achievements during Phase II –

Ozone continued to be pursued as a strategy for initial sterilization of the PBRs, headers, and salt water. Compared to horizontal, large volume, foil-mixed PBRs, smaller, simpler vertical PBRs had lower total volumes to be treated per PBR, fewer cryptic spaces, and no internal moving parts, making it easier to ensure that the entire volume was exposed to therapeutic concentrations of ozone. Multiple demonstrations with contamination control (CC) broth and with cultivations in salt water showed that ozone could sufficiently sterilize new bioreactors and salt water for extended ethanol cultivation. In spite of these successes, additional CIP strategies

needed to be developed for use on used PBRs in between batches. Residual biomass and inorganic deposits remained on the plastic post-drain, making it challenging for ozone to reach all PBR surfaces. Thus, liquid chemical CIP steps were pursued to remove residual biomass prior to re-sterilization (Figure B-10).

Because of continued deterioration of ethanol production rates during 2013, residual biomass and inorganics on the PBRs post-drain, and interactions between ozone and bioreactor materials, new CIP methods were investigated to define protocols that increased effectiveness and consistency between batches and integrated CIP/sterilization with system materials and design. To evaluate the effectiveness of CIP methods, the concept of maximum acceptable contaminant level (MACL) was applied—MACLs are similar to the EPA's drinking water standards and were set at $<10^4$ total bacteria and $<10^3$ for common ethanol consumers measured 24 hours post-inoculation. After multiple iterations, a final robust method was determined.

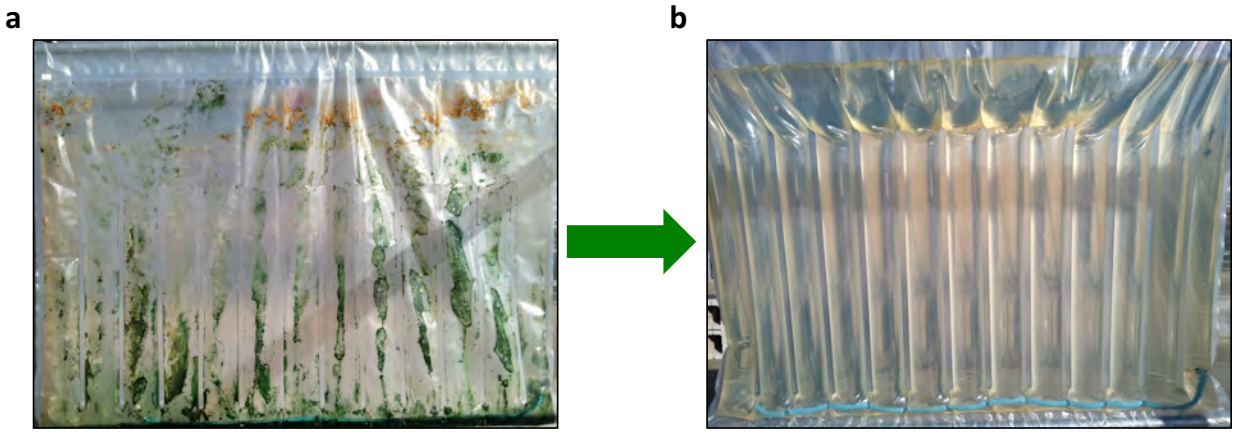


Figure B-10. PBR after a typical (~20-30 day) batch highlighting residual organic and inorganic deposits remaining on the plastic post-drain (a). Clean PBR after liquid chemical CIP.

Subtask B.2 Pilot plant construction and associated fees

Completed April 2013

In order to better control project risks, a phased approach was used in Budget Period 2. Phase 1 included installation of the primary infrastructure to operate the biorefinery on a limited basis, excluding downstream ethanol purification processes and equipment, and included the installation and operation of 60 PBRs. The 40 PBRs were operated continuously for 60 days. At the conclusion of the 60-day operating period, a go/no go/redirect discussion was held between Algenol and DOE based on the following Phase 1 metrics: If after 60 days of continuous operation, 75% of the PBRs have maintained their ethanol concentration and the mixing system is still operating, Algenol will proceed to Phase 2. If there is a greater than 25% failure rate due to contamination (not mechanical issues), additional studies may be pursued in Phase 1 to further address any remaining issues.

Prior to commencement of construction of the IBR, Algenol hired a consultant to provide training pertaining to compliance with the specific rules required to perform work on a contract from the DOE and the Recovery Act. In order to work on the project, Algenol required the general contractor and all sub-contractors to attend the meeting and receive formal training for Davis Bacon reporting and preparing all documentation required by the DOE for reimbursement. In addition, they received training to prepare for the external audit that was to be conducted during the project.

Phase 1

The permit submittal package for IBR construction was submitted to the Lee County Building and Permitting Department in July 2011. Due to the phased approach to Task B, construction of the IBR was also divided into phases. The first phase focused on installing the site electricity, water, sewer, stormwater management, buildings, roads, parking and landscaping (Figure B-11). This infrastructure was necessary to operate the IBR sufficient to conduct testing at the scale needed to de-risk and move into Phase 2.

Figure B-11. First day of IBR construction.

In August 2011, the site surveying and soil testing were completed on the IBR site. The silt fencing was installed on the site, and GCM Contracting mobilized earth moving equipment to the IBR site and commenced soil moving. The first focus of construction was clearing the area where the lab/office/shop and process pavilion were to be located along with raising the building floor elevation above the 100-year flood elevation required by local building codes. Just over 30,000 cubic yards of clean fill were brought in and compacted as the base for the two structures to be constructed.

Upon completion of the setting of the grade elevation for the buildings, the buildings' dimensions were laid out. Then, excavation was completed for the footers for the pre-fabricated steel buildings and the appropriate rebar was installed as well as the bolts for securing the building columns to the footers. A total of 40 cubic yards of concrete was poured for the building footers.

All underground piping and conduit were installed before the concrete for the floors for both buildings were poured. Once the underground work was completed, approximately 40 cubic yards of concrete were poured for the floors of both buildings. All concrete used on the site was tested and certified for its compressive strength.

Concurrently with the construction of the buildings, rough grading site work was completed. This focused on setting the elevations of the stormwater retention areas, installation of underground stormwater piping, and building the controlled site stormwater outflow structure.

At the beginning of November 2011, the prefabricated buildings arrived on site, and the erection of the process pavilion began. GCM also started the erection of the prefabricated

greenhouse located next to the main building. In early December 2011, the erection of the two prefabricated buildings was completed, and construction started on the interior of the main building. The pipe trough and floor of the process pavilion were also started.

At the end of January 2012, Florida Power and Light finished installing the site power loop, set the transformer and turned on the permanent power to the site. By the end of February 2012, the interior of the main building was complete and being readied for final inspection. All of the landscaping was completed, the sub base, binder and asphalt surface course were installed on the roads and parking lot. The certificate of occupancy was issued by Lee County in March 2012. Algenol personnel then worked to install the necessary water piping, filters, tanks and PBR systems to compile the data necessary to conduct the Phase 1 gate review (Figure B-12).

Figure B-12 IBR completed.

Cardno ENTRIX developed the package that was submitted to the Florida Department of Environmental Protection (FDEP) for authorization to build an injection/supply well on the site. Algenol submitted the Direct to Ethanol® process waste water quality analysis to the FDEP for classification. FDEP classified the water as wastewater from an aquaculture operation. Therefore, the well was classified as a Class 1 injection well. This required the effluent to be injected below the Underground Sources of Drinking Water (USDW) which in this particular case was at a depth from the land surface to approximately 1,400 feet. The FDEP approved well was designed to inject/supply water between 1,600 and 1,800 feet below land surface. A monitoring well was installed at 1,200 feet below land surface. The monitoring well is sampled monthly to determine if injection water is passing through the barrier between the injection zone and the USDW zone. In May 2012, the final contract for the aquifer storage and recovery well was finalized and the initial preparation for well drilling commenced. Southeast Drilling Services was awarded the contract. The well took approximately 11 months to complete.

Regulatory approval of ethanologenic strain

In order to utilize Algenol's modified AB1-based ethanol producing strain in the IBR, Algenol needed to receive regulatory approval for the IBR facility and strain. Algenol received approval to conduct research and development at the IBR and also received a Consent Decree from the US Environmental Protection Agency (EPA) that allowed the Company to initiate commercial operations at its Fort Myers, FL facility as requested in the submitted Microbial Commercial Activity Notice (MCAN). The Project provided an impetus for the Company to expand its broad, proactive and transparent approach to engaging regulatory agencies on both the well-defined regulatory requirements described in section A.7 above and, especially with respect to commercialization of a cutting-edge technology utilizing genetically enhanced algae.

Florida regulatory overview – In addition to obtaining the aquaculture license for the project the Florida Department of Agriculture and Consumer Services (FDACS) required additional strain specific approvals to deploy the Company's enhanced algae. To date, several strains have been approved for deployment at the biorefinery including the strain identified for potential commercial deployments, AB1. These approvals are based on an initial screening paradigm designed to eliminate potentially harmful strains as well as a series of robust environmental studies that were designed, and in some cases conducted, with significant oversight from FDACS.

Executing the Project required Algenol to establish an early commitment to documenting the environmental safety of the enhanced algae starting with the identification and development of a candidate strain in the laboratory. Candidate strains are rigorously screened for their performance characteristics with respect to ethanol and biomass production inside the PBR and for environmental considerations to ensure they are non-toxic, non-invasive and are not plant pests. The Company's proprietary screening paradigm is augmented by a battery of environmental studies that each strain is subjected to at the appropriate stage of development. Along with developing a history of safe use, these studies have repeatedly yielded significantly similar results that demonstrate the enhanced algae's environmental and human health safety. The environmental studies include:

- Non-invasiveness testing to determine the enhanced algae's ability to thrive in the event of a spill onto soil or a variety of natural water types;
- Whole genome sequence analysis and annotation in order to perform genome-wide screening to show the absence of cyanotoxin pathway genes; and
- Qualitative analysis of working cultures, both biomass and media, to confirm the absence of cyanotoxins using high performance liquid chromatography (HPLC).
- Assessment of horizontal gene transfer potential. This study demonstrated the inability of the enhanced algae to transfer the introduced fermentation pathway enzymes to other organisms in the event of a release into the environment.

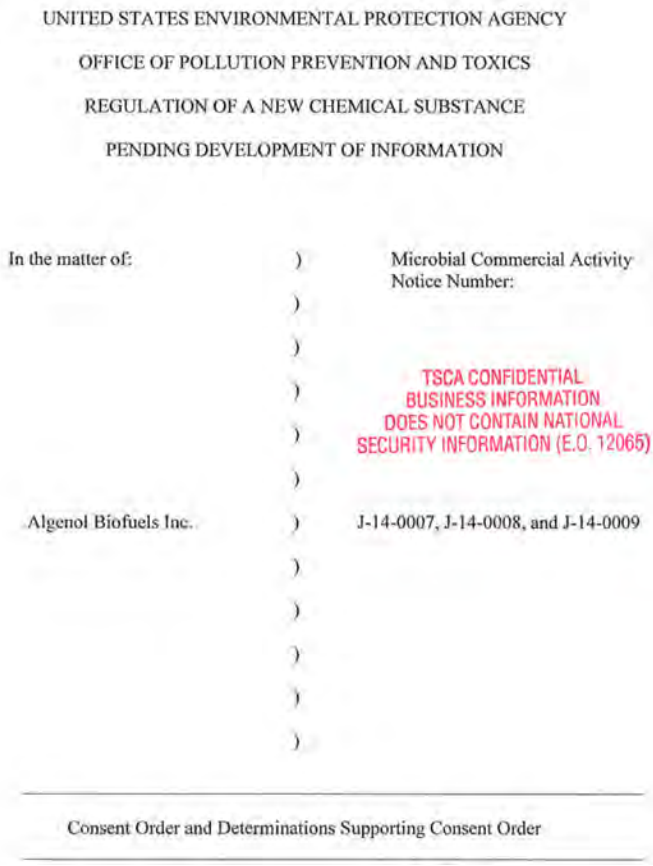
The most important of these studies, which were conducted after confirming an inability to produce toxins, were the non-invasiveness studies designed with input from FDACS and their academic advisors showing that the enhanced algae are not able to survive in natural conditions. The experiments' design protocols required adding very high densities of enhanced algae to 5 different water samples collected at various points starting with a ditch near the facility and culminating at the Gulf of Mexico, thus including fresh water, brackish water and salt water specific samples. Following inoculation, nutrients and oxygen were provided to the samples to provide optimal growing conditions. The high density of enhanced algae yielded a pronounced green coloration of the water incubation bottles at the time of inoculation. In all incubations, the test organism failed to proliferate and immediately began to disappear such that

the green coloration was cleared within one week. Native algae present in the water samples flourished and formed dense mats, indicating that simulated in-situ culture conditions were amenable to algal growth. The presence of the enhanced algae during the lab incubation of the water samples was monitored by the polymerase chain reaction (PCR) with specific primers for genomic and plasmid DNA of the test organism. Consistent monitoring of the samples showed a rapid initial fatality rate and within 60 days for all cultures the enhanced algal strain was completely undetectable.

Building on the strength of the environmental studies, Algenol took the additional step of obtaining an exemption for AB1 from certain special permitting requirements for non-native species, which was done through a formal rulemaking process and is codified in Florida statute. That exemption can be found in Florida Rule 5B-57.011(5), Algenol's commercial strain is identified therein as *Cyanobacterium sp*. (Strain AB1).

US federal regulatory overview – During the early stages of the Project and prior to commercial operations, the Company operated under certain research and development and contained structure exemptions established by the Environmental Protection Agency pursuant to Toxic Substances Control Act (TSCA) regulations. As the Company transitioned to pilot-scale production and commercial demonstration activities it was necessary to file a MCAN consistent with TSCA. The process for completing the MCAN and negotiating the corresponding Consent Decree was a comprehensive process that took more than a year, starting with pre-submission notification meetings between Algenol and EPA to identify submission requirements. The MCAN Algenol submitted described every aspect of the overall process in addition to highly detailed information regarding the host algal strain and the genetic enhancements executed by Algenol's scientists. Also included were the many environmental studies Algenol had conducted in order to obtain approvals from the state of Florida. Following the initial submission, the EPA made multiple additional data requests.

On December 12, 2014, Algenol and EPA agreed on a Consent Decree that allowed the Company to initiate commercial operations at its Fort Myers, FL facility as requested in the submitted MCAN (Figure B-13). Also negotiated into the Consent Decree is a roadmap for future MCAN approvals including reasonable information EPA requested in order to approve an MCAN for a commercial scale project.



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

OFFICE OF POLLUTION PREVENTION AND TOXICS

REGULATION OF A NEW CHEMICAL SUBSTANCE

PENDING DEVELOPMENT OF INFORMATION

In the matter of:	)	Microbial Commercial Activity Notice Number:
	)	
	)	
	)	TSCA CONFIDENTIAL BUSINESS INFORMATION DOES NOT CONTAIN NATIONAL SECURITY INFORMATION (E.O. 12065)
	)	
	)	
Algenol Biofuels Inc.	)	J-14-0007, J-14-0008, and J-14-0009
	)	
	)	
	)	
	)	
	)	

Consent Order and Determinations Supporting Consent Order

Figure B-13. EPA Commercial Microbial Activity Notice.

Phase 2

Construction efforts in Phase 2 focused on water processing and pumping, downstream biomass and ethanol processing, and cultivation systems. All construction efforts in Phase 2 were conducted by Algenol personnel with some sub-contracting to GCM and our preferred electrical sub-contractor. Once the IBR process pavilion was finished, the IBR team installed the water system. The water system was designed to take incoming water from the salt water well or, as a back-up, salt water trucked to the site, and pass it through a series of filters ranging from 20 microns to 0.2 micron. This filtration removed all biomass and most contaminants in the water. This water was stored in tanks that supplied the ultrafiltration (UF) unit. The water was then passed though the UF system (0.02 micron) and stored in the process water tanks. The water was then treated with ozone to ensure the water remained free of microorganisms. This water was fed into the process water supply loop. The process water supply loop ran through the facility to the greenhouse, the lab and the PBR field and returned back to the storage tank. This ensured the water was always contacted with the ozone. A large air compressor and compressed air supply system was installed that serviced the IBR complex. Potable water was also piped throughout the IBR complex.

A biomass harvest system was installed. This system consisted of holding tanks for incoming culture, a centrifuge, a microfiltration skid and holding tanks for biomass and ethanol-rich supernatant.

A motor control center was installed to provide power to all systems in the process pavilion and PBR fields. A large diesel back-up generator was installed and connected to an automatic transfer switch. All critical equipment was connected to the generator.

A 30 ton liquefied CO_2 tank was installed to supply CO_2 to the greenhouse and PBR fields.

With the upstream and biomass harvest systems installed, the bioreactor systems could be installed and operated at the IBR. Algenol followed a 10x scale up strategy with respect to its PBR cultivation systems. All installed cultivation systems were built as standalone with respect to installed equipment and control systems. Each system had installed blowers for air supply, tanks for nutrient supply, filtration systems for air, CO_2, nutrients and water, piping and control valves, and a PLC for systems control.

40-block – The first system to be installed was a 40 PBR system. This system consisted of 40 flexible film PBRs installed as a single operational unit. The PBRs were the first generation PBR design (VIPER1) toll manufactured for Algenol. These PBR were 3' x 3' and held 20 liters of culture volume.

400-block – The next scale was a fully integrated block of 400 vPBRs (Figure B-14). This system consisted of a block with four rows of 100 VIPER1 PBRs. These PBRs were 3' x 3' and held 20 liters of culture volume.

Figure B-14. Ethanologenic cultures at the IBR 400-block.

4000-module – The 4000-module represented the next leap in scale, with five blocks of 800 VIPER1 PBRs as operational units (Figure B-15). This system also had a scrubber tower for the recovery of ethanol in the vapor stream as oxygen is purged from the system.

Figure B-15. Ethanologenic cultures at the IBR 4000-module.

1.8-acre module – The final scale for demonstration of commercial ethanol production for this grant was a PBR array of 1.8-acres (Figure B-16). The installation of 1.8 acres required more infrastructure to be installed at the IBR to support this next generation system. A small process pavilion was installed (4,000 sq. ft.), a new power transformer, a new fire hydrant, a new process pad, scale-up pad, and supports for the array. A total of 15 PBR blocks made up the array, including 8 blocks of 408 integrated VIPER 2.3 PBRs, 5 blocks of 408 integrated VIPER 2.3, and 2 blocks of 200 VIPER 3.1 airlift systems.

Figure B-16. Ethanologenic cultures at the IBR 1.8-acre module.

With the 1.8-acre array producing ethanol, there was enough feed into the downstream system to support the pilot scale VCSS and ethanol dehydration systems. The VCSS column

and reboiler were fabricated at a shop and shipped to the site for installation. The other equipment used in the unit were procured directly without need for custom fabrication. Algenol personnel installed the VCSS column, reboiler and all piping, tanks, valves and PLC to complete the system.

For the second stage of ethanol purification a standard Stripper, Rectifier and Dehydration (SRD) system was installed to dehydrate the ethanol to fuel grade specifications. Algenol enginners and technicians built this unit at the IBR.

A 2,000 gallon ethanol storage tank and a 500 gallon gasoline tank were installed for finished product storage and denaturant respectively.

Subtask B.3 Personnel hiring, training, and documentation

Completed July 2015

The hiring for the IBR was done over a several year period as the facility expanded its operations from 2011 to 2015. The project started with a core group of staff. As the project progressed and went through its scale up protocol from 40 PBRs to 400 PBRs to 4,000 PBRs to 1.8 acres of PBRs, the need for additional employees increased. The operation of the 1.8 acre site and downstream processing equipment required 7 day per week operations and some shift work.

Algenol recruited the IBR Plant Manager from its existing employee pool. Likewise, the plant engineer was assigned from the Company's engineering department. All financial functions of the IBR were performed by Algenol's existing finance team. Algenol hired several people in its finance and administrative departments to manage the increased work load from IBR activities.

Integrated Biorefinery Staff Levels	
July 2012	9
July 2013	11
July 2014	22
July 2015	23

Several Algenol personnel were assigned to the IBR to work in biological operations. Additional biological operations personnel were hired and subsequently trained at the PDU. IBR maintenance and equipment operators were all hired from the local talent pool.

Upon completion of training, the IBR staff and the staff at the PDU collaborated to write SOPs for all IBR operations. As the IBR scale up protocol was implemented, new SOPs had to be developed for each scale. Since each scale had new equipment sized for that production unit, new SOPs had to be developed for each increase in scale.

Algenol's Environmental, Health and Safety (EH&S) committee planned, directed and implemented EH&S programs, procedures and policies at the IBR to ensure occupational, health and environmental safety compliance. The committee addressed, among other issues, overall EH&S procedures, safety training, lock-out/tag-out procedures, confined space entry protocols, OSHA's laboratory and facility safety standards, requirements for preparation of Chemical Hygiene Plans, Hazard Communication Standard, Occupational Exposure to Hazardous Chemicals in Laboratories and facilities standards, and limits on airborne contaminants such as carbon dioxide and ethanol.

Subtask B.4 Shake-down runs

Completed December 2014

Algenol established commissioning and operation teams in addition to the engineering teams that worked in tandem to develop, write, and approve commissioning and startup policies,

standard operating procedures, and other documentation required for the commissioning, startup, and operation of the plant.

Commissioning activities commenced when a specific whole system was mechanically, electrically, and instrumentally complete. The commissioning team coordinated closely with the QA/QC personnel to communicate observations and experience with the quality of equipment, vessels, parts, services, and construction as the commissioning of the plant moved forward. The communications efforts documented and corrected issues as well as noted positive results. Commissioning personnel took an active role on quality efforts to resolve issues, but it was the responsibility of Algenol's QA/QC personnel to provide the ultimate documented and implemented solutions.

Meetings at the beginning of each shift reported progress and any unresolved issues with commissioning and general shift transition communications. Issues and opportunities for follow-up were documented utilizing established procedures for safety, maintainability, operability, or otherwise with action items, responsibilities and accountabilities assigned.

Representatives from the IBR Biology team and the Maintenance team were also involved in specific commissioning and startup activities alongside commissioning and operations teams. Personnel were sometimes on multiple teams at the same time to facilitate interactions and communication. Each team participated in the initial commissioning efforts and helped develop the necessary commissioning policies, procedures, and equipment checkout requirements.

Once the Facility was online, the IBR Operations team was responsible for daily operations of the shake down runs. The shake down runs consisted of 2-3 weeks of complete operations with water only. This allowed the teams to safely run through each SOP multiple times to ensure all systems operated as designed. It also allowed the operations team to ensure all personnel were trained properly.

Algenol followed a 10x scale up protocol from 40 PBRs to 400 PBRs to 4,000 PBRs to 1.8 acres of PBRs. Each new system went through a commissioning and shake down period. Once all shakedown runs were completed and all PBR blocks were ready for production, the system was inoculated, and the facility entered operational mode and produced ethanol over an extended period of time.

Subtask B.5 Techno-Economic Analysis

Completed May 2017 (evergreen analysis)

A strong commitment to Techno-Economic Analysis (TEA) has been an integral part of Algenol technical portfolio from company startup in 2006. As a novel technology, the earliest versions of TEA for the Direct to Ethanol process were rudimentary because so many of the engineering and biological systems were first-of-kind. However, the TEA results were compelling enough to attract private equity funding sufficient to enable proof of concept and provide the matching funds needed for the current proposal. The underlying physical system that formed the basis for the TEA also formed the basis for the Life Cycle Analysis (LCA) which showed that Direct to Ethanol could provide a very significant reduction in CO_2 footprint compared to gasoline, well beyond the requirements for this DOE project. The LCA work, which has produced two peer reviewed publications, will be discussed in detail elsewhere in this report.

TEA analysis, combined with Algenol's financial model, provides an economic assessment of the viability of the Direct to Ethanol technology under various scenarios for future economic conditions, primarily oil prices, but also including government incentives for carbon footprint reduction for transportation fuels. In that same context, the TEA with its Capital Expense

(CAPEX) and Operating Expense (OPEX) goals provides research guidance for both performance improvements and cost reductions. The TEA has been used in that manner from Algenol's beginnings and throughout the execution of the IBR project. There are examples of this in every aspect of the IBR operation, some of which will be discussed below.

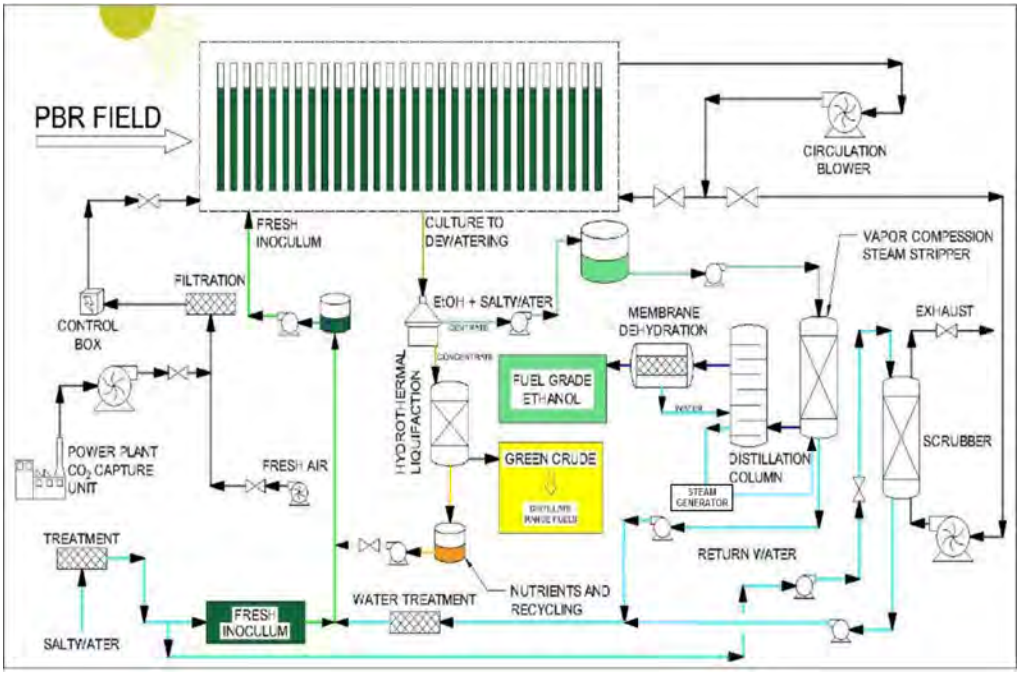


Figure B-17. Block flow diagram that forms the basis for the TEA. Final products are fuel grade ethanol and green crude. A Combined Heat and Power (CHP) unit (not shown) provides the heat and power for the operation.

The system being modeled in the TEA is depicted in Figure B-17. The scale for the system is chosen as 2000 acres. There will be an optimum size that, dependent of some of the details of the modeling, will be between 3000 and 7000 acres. The reason there is an optimum is that much of the downstream equipment has a normal positive scaling factor (meaning the bigger the better), while the upstream has a negative scaling factor due the network of piping for both liquid and gas transport. 2000 acres is chosen as a reasonable size for the TEA study and for commercial deployment, after consideration of limitations on the sizing of some elements of the downstream equipment, wherein the scaling factor would be lost. The system is also modeled as the nth plant with the learnings from the IBR deployment and operation included, as well as the learnings from the various project teams involved in the execution of the DOE project.

Figure B-18 depicts the division into technology areas (e.g., area 400, Ethanol Separation), each of these being associated with a corresponding project team or teams. For the 2000 acre commercial model, these system areas were subjected to detailed design, energy demand, and economic analysis. Thus, in the TEA model, there are 11 TEA modules corresponding to Areas 100–1100. Each module is fully-loaded (all materials, labor, and energy demand) and feeds into a master sheet for system integration and development of the input (CAPEX, OPEX) for the Financial Model. The main system goal is cost reduction leading to overall economic optimization of the integrated system. Because of the balanced electrical and heat requirements, a CHP system (in area 1100) provides optimal economics and is also important for achieving a low carbon footprint. Not shown is area 9000 which covers fixed costs for the plant (e.g., insurance and other items not associated with a particular module).

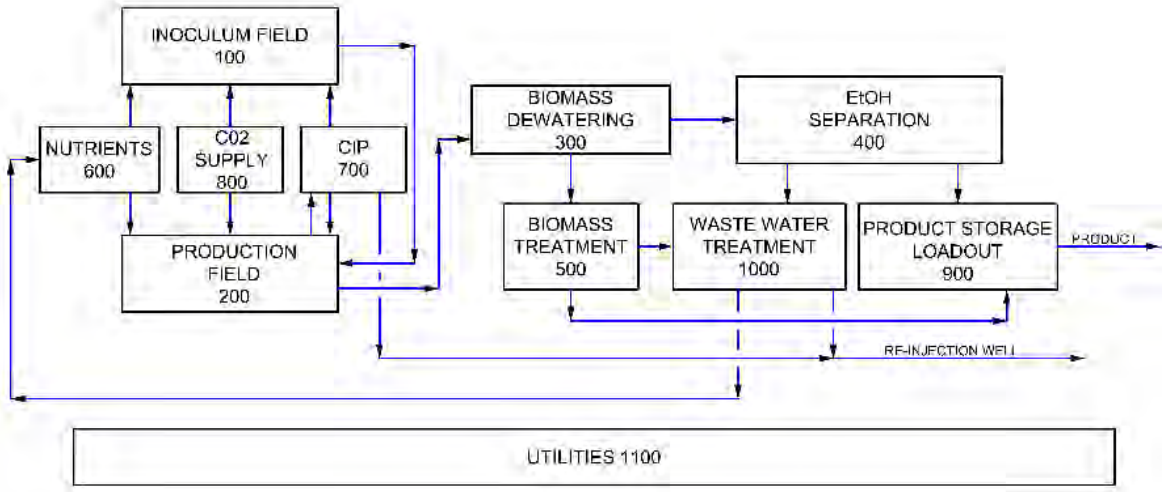


Figure B-18. Plant Block Flow Diagram for Engineering TEA.

The overall structure of the system is shown in Figure B-19. As indicated in the figure, the project teams make input to the 11 technology modules (plus area 9000), basically serving as the subject matter experts for the TEA and the TEA leader. The TEA Leader collects and validates the information from the teams and incorporates that information into a TEM Master Sheet, which ultimately produces CAPEX and OPEX input for the Financial Model under various commercialization scenarios. The Master Sheet is also a primary input for Life Cycle Analyses conducted in collaboration with Georgia Tech. Except for the Financial Model, this entire process is owned by Algenol Engineering as part of the IBR project, and reviewed periodically by the Project Management Team. It is also reviewed quarterly by Algenol's Joint Technical Committee, and especially by our major industrial partner, Reliance Industries Limited (RIL). RIL makes input to the TEA process, structure, and commercialization scenario development. The Financial Model is owned by the Financial Team, but reviewed in much the same manner as the TEA.

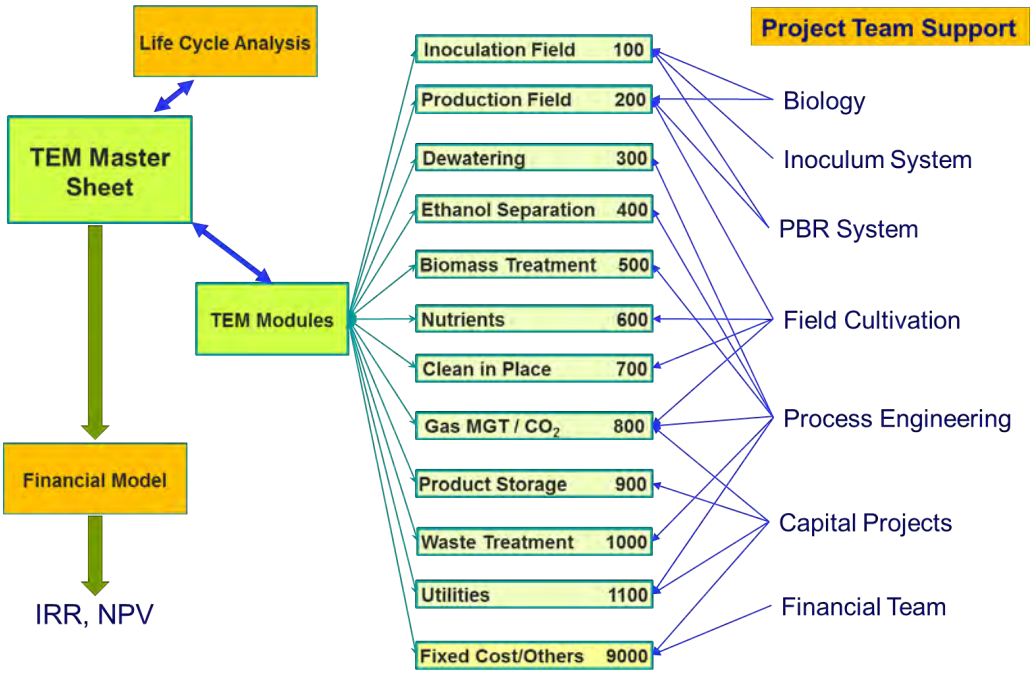
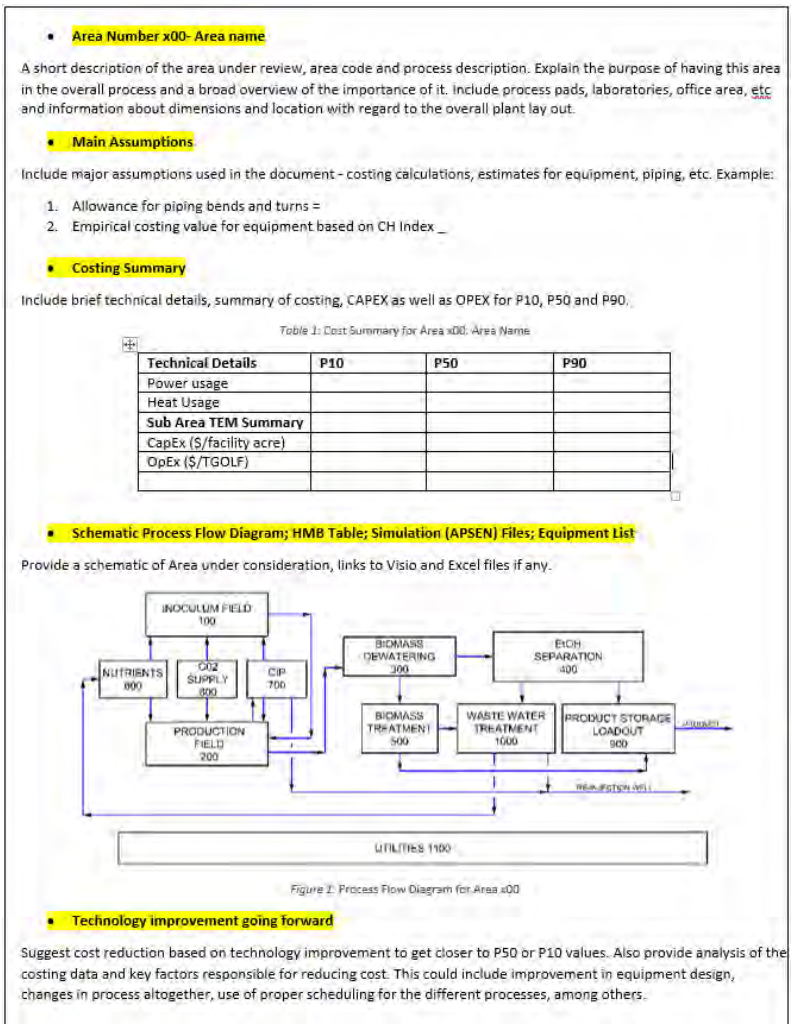


Figure B-19. Techno-Economic Model structure.

In terms of final reliability assessments for the various areas, areas 200, 400, 700, 800, 900, 1000, and 1100 are viewed by the project team and Algenol management to be highly reliable in terms of the performance and economic aspects of their operation. Areas 100, 300, and 600 are viewed to be reliable, but with remaining questions in terms of technology choices and costs. Only one area, biomass treatment, is judged to have low reliability mainly because Algenol has built but not commissioned the hydrothermal liquefaction (HTL) unit, nor have we conducted HTL runs with actual feeds from the IBR. Pacific Northwest National Laboratories (PNNL), under contract from Algenol, has conducted HTL experiments with wild type AB1 from Algenol and obtained reasonable conversion yields (34%) and a high quality product (gasoline, diesel, jet fuel range molecules) after upgrading/hydrogenation. RIL has extensive experience with HTL technology and operates a number of HTL units of various capacities. It is for those reasons that Algenol elected not to devote significant time and effort to independently developing HTL for the waste biomass, which is a relatively minor component in our projected fuel output (about 15% of the TGOLF values for commercial strain).

The backup TEA documentation involves area descriptions in the form of a template depicted in Figure B-20. The template includes an introduction about each area and a basic process description. That is followed by a description of the main assumptions used in the calculations. The costing summary for both CAPEX and OPEX is presented in a P10, P50, and P90 format, which represent the probabilities for the listed cost outcomes based on subject matter expert input. The P90 values represent cost projections (or performance projections in some instances) that have a 90% probability of being achieved on the time scale of commercialization. Thus, P90 values are the most conservative projections and tend to be close to currently achieved performance and cost. P50 values are 50% probabilities, representing reasonable expectations for cost and performance. These values are most commonly used for judging the economic status of the project. P10 values are 10% probabilities and are often referred to as stretch targets. We require that those targets be physically reasonable, i.e., thermodynamically possible for example.



- **Area Number x00- Area name**

A short description of the area under review, area code and process description. Explain the purpose of having this area in the overall process and a broad overview of the importance of it. Include process pads, laboratories, office area, etc and information about dimensions and location with regard to the overall plant lay out.

- **Main Assumptions**

Include major assumptions used in the document - costing calculations, estimates for equipment, piping, etc. Example:

1. Allowance for piping bends and turns =
2. Empirical costing value for equipment based on CH Index _

- **Costing Summary**

Include brief technical details, summary of costing, CAPEX as well as OPEX for P10, P50 and P90.

Table 1: Cost Summary for Area x00: Area Name

Technical Details	P10	P50	P90
Power usage			
Heat Usage			
Sub Area TEM Summary			
CapEx ($/facility acre)			
OpEx ($/TGOLF)			

- **Schematic Process Flow Diagram; HMB Table; Simulation (APSEN) Files; Equipment List**

Provide a schematic of Area under consideration, links to Visio and Excel files if any.

Figure 1: Process Flow Diagram for Area x00

- **Technology improvement going forward**

Suggest cost reduction based on technology improvement to get closer to P50 or P10 values. Also provide analysis of the costing data and key factors responsible for reducing cost. This could include improvement in equipment design, changes in process altogether, use of proper scheduling for the different processes, among others.

Figure B-20. TEA documentation template.

Next is the technical data represented typically by process flow diagrams, heat and material balance (HMB) tables, and links to relevant technical calculations: ASPEN Plus, Excel files, etc. The final section deals with possibilities for technology improvements. Those could include both optimization of the current design, but also new concepts that could be developed in the future. This area is strongly coupled to the P10, P50, and P90 estimates described above. Contributors and reviewers to this document are denoted at the bottom of the template.

The HMB is an important element for both the TEA and LCA assessments. An example is shown in Figure B-21 for the “final” P90 assessment for the IBR project, the key parameter being the productivity assessment of 5600 TGOLF (4800 GEPAY).

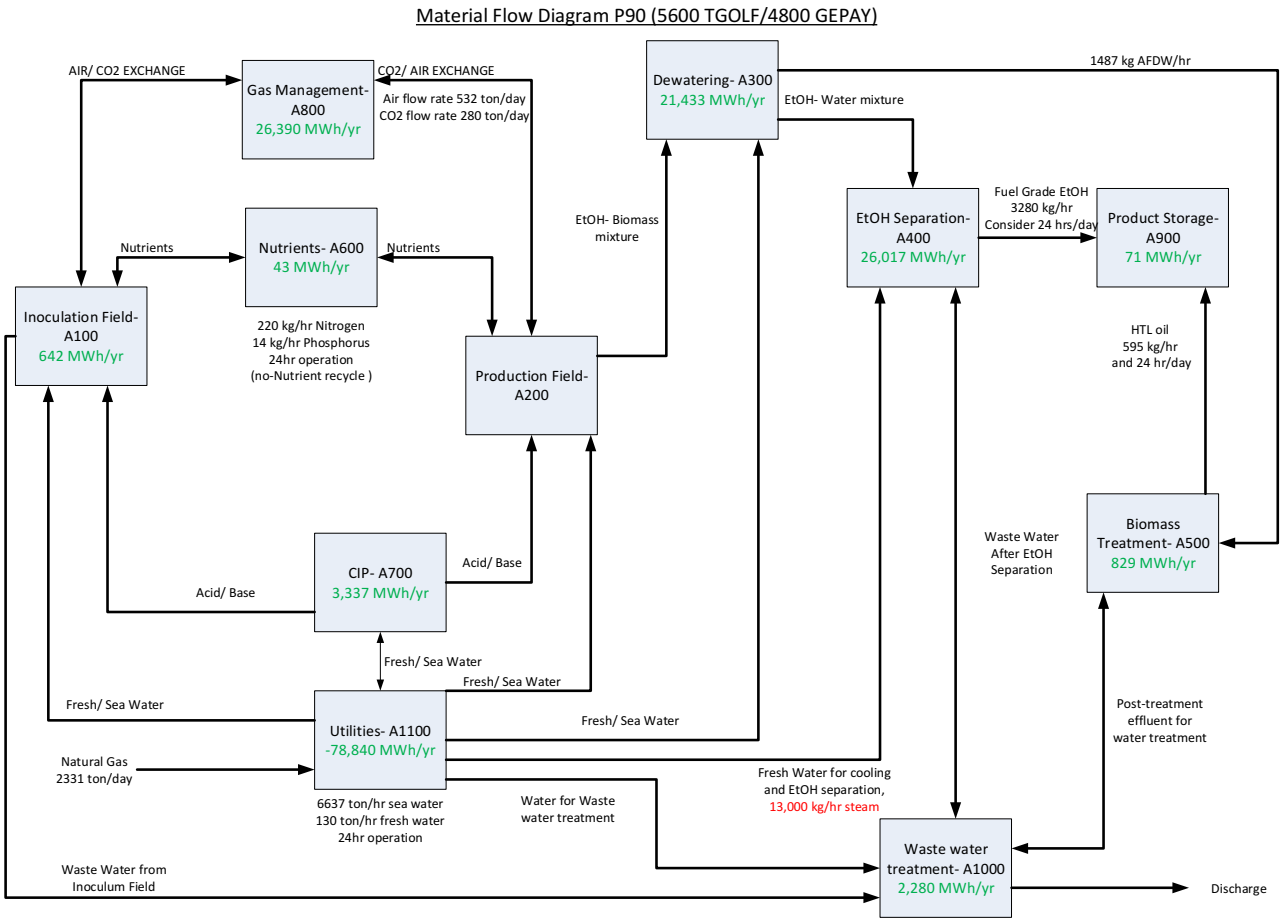


Figure B-21. Material Flow Diagram representing the HMB with P90 values for the various parameters entering the calculations for the TEA calculations.

The layout for the 2000 acre plant is shown in Figure B-22. This layout forms the basis for the piping networks and other aspects of the design. The CO_2 management module treats the CO_2 as being available at the plant boundary limits as either a flue gas or a concentrated stream.

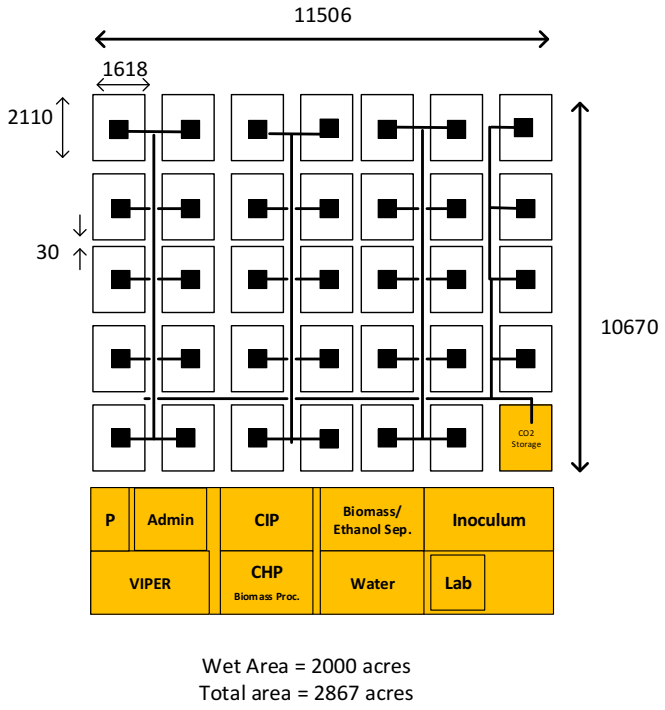


Figure B-22. Model layout for 2000 acre Algenol plant. Labeled dimensions are in feet. Each open square is about 60 acres. The solid square represents the field process pad which contains cultivation operation equipment for one 60-acre module, including aeration gas blowers, filters, harvest system, etc.

In the photobioreactor field, the base Unit is an array of 90 VIPER PBRs interconnected with liquid and gas piping. The wet area of the Unit is defined as the footprint area directly under the water-filled PBR array. A block consists of 46 such base units and each block measures 2.5 acres. One module consists of 24 blocks, arranged symmetrically with a Field Processing Pad in the middle. The 2000-acre facility consists of 34 modules, along with all the piping, facility utilities, labs and downstream equipment.

Photobioreactor system costs (P10/P50/P90)

The PBR system includes the PBR (with gas diffuser & tubing kit), Support Frame, and Piping System. The major assumptions and cost summary of the PBR System is given in Table B-1. P90 is based on the design used in Algenol IBR (1.8-acre plant); P50 is the R&D target for PBR system with improved design and also with cost reduction from piping system optimization; P10 is the long term research goal for a self-supported PBR system, which has been designed but not built. The cost estimations listed in Table B-1 were based on quotes for 2000-acre ethanol plant with 7 million PBRs.

The PFD of Area 0200 (PBR Field as example) and Piping system inside of the 60-acre module are shown in Figure B-23 and Figure B-24. The piping network inside the 60-acre module and interconnection of modules are designed and optimized for filling and harvesting 2 blocks (2.5 acre per block) from each 60-acre module at same time, and the gas piping is designed to achieve an overall pressure drop of 5 psi.

Table B-1. Cost summary for PBR system.

Area 0200 Module Technical Details			
	P10	**P50**	**P90**
Major Design Assumptions	2000-acre	2000-acre	2000-acre
PBR Bag	Self-supporting PBR made of thermoset	VIPER 4.0	VIPER 3.2
Support Frame	Limited steel rods for anchoring PBRs to the ground	Arched tube for support	3-post metal frame
Piping system	Optimized piping design, block sizing, mixed alternatives	Various pipe design based on current alternatives	Reference to 1.8-acre design
PBR Bags life time (yr)	12	8	6
PBR Bags per acre	3268	3268	3268
PBR Costing Summary (per 10-ft)			
PBR Bags + tubing kit+ diffuser, ($/PBR)	7	9	12
Support Frame, ($/PBR)	0	7	9
Piping inside Module, ($/PBR)	3	3	*12*
Valve & Sensor inside Module, ($/PBR)	2	4	4
Total installed PBR system cost ($/PBR)	10	23	37
Area 0200 TEM Module Summary			
CAPEX, $/acre	38,560	73,860	119,610
OPEX, $/acre per year (PBR bags amortization)	2,400	4,550	8,300
OPEX, Man-hr per year	70,080	116,800	210,240
Long Term CAPEX, $/acre	18,950	47,710	83,660
Short Term CAPEX, $/acre	19,610	26,140	35,950

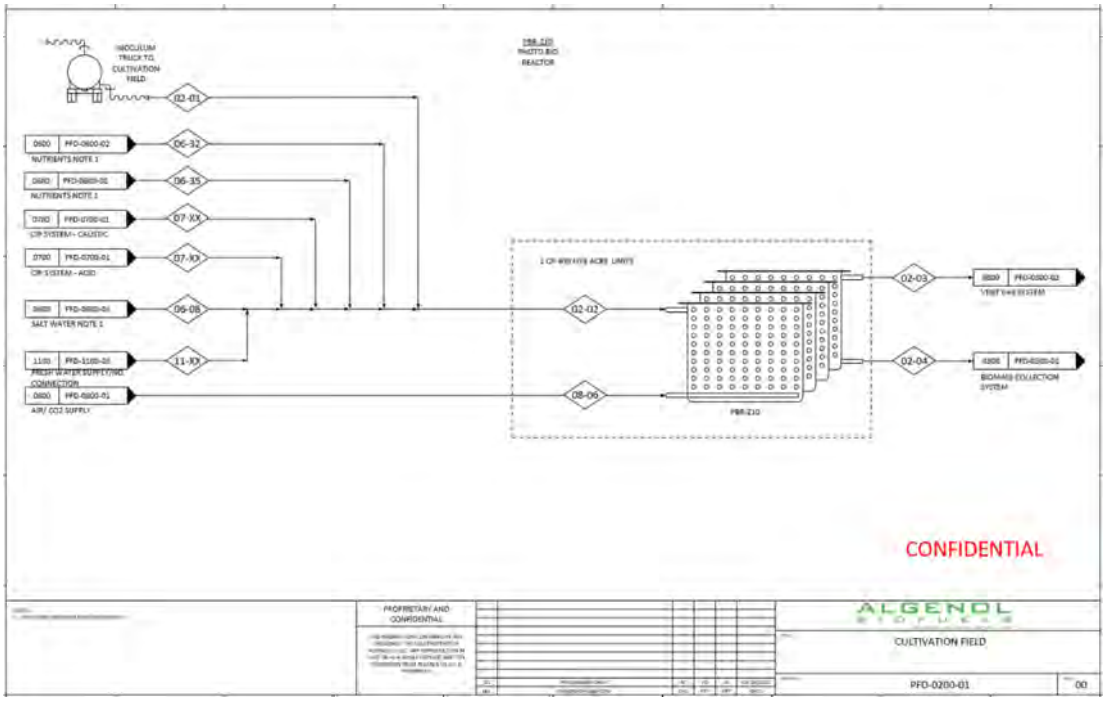


Figure B-23. The PFD for Cultivation Field.

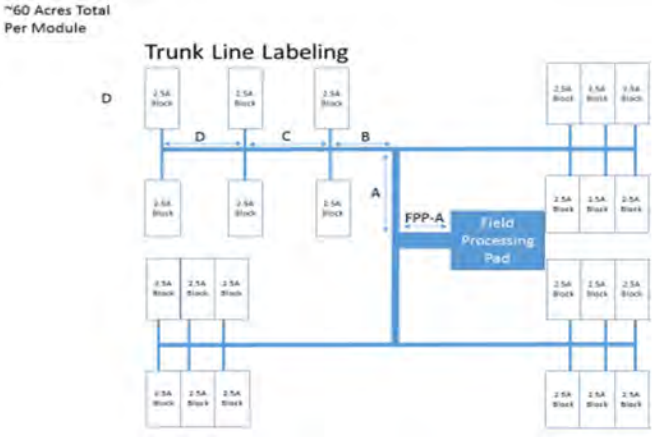


Figure B-24. The piping system for 60-acre module.

TEA CAPEX/OPEX

CAPEX and OPEX calculations are based on each AREA TEA Module. The basic design is for a 2000-wet acre plant. Long term CAPEX is calculated for all Areas and the time period considered for depreciation is 24 years. Short term CAPEX is calculated for the PBRs for a time period of 12 years (P10), 8 years (P50) or 6 years (P90).

Table B-2. TEA CAPEX summary table for 2000-acre ethanol plant.

Long Term CapEX	24 yr		P10	P50	P90	Analytical Reliability Assessment
	Area	Area code	CapEX ($/facility-acre)	CapEX ($/facility-acre)	CapEX ($/facility-acre)	
	Innoculum	Area 0100	421	919	1223	Medium
	Production Field	Area 0200	14,400	38,600	93,600	High
	Dewatering	Area 0300	2400	3400	6400	Low
	EtOH Separation	Area 0400	7,036	10,060	24,170	Medium
	Biomass Treatment	Area 0500	6,750	8,437	15609	Low
	Nutrients	Area 0600	370	370	1312	Medium
	CIP	Area 0700	2519	4596	7064	High
	Gas Management/CO_2	Area 0800	5,153	17,206	22,775	Low
	Product Storage Loadout	Area 0900	1869	1868	1869	Medium
	Wastewater Treatment	Area 1000	3950	7400	3500	Medium
	Utilities	Area 1100	11301	11301	11301	High
	Others	Area 9000				
	Sum.		56,169	105,159	188,824	
			P10	P50	P90	
Short Term CapEX	6 to 12 yr		12	8	6	
	Area	Area code	CapEX ($/facility-acre)	CapEX ($/facility-acre)	CapEX ($/facility-acre)	
PBR Bags	Innoculum	Area 0100	16.64	113	173	Medium
PBR Bags	Production Field	Area 0200	21,600	28,800	39,600	High
	Sum.		21,617	28,913	39,773	
Total CapEx			77,786	134,071	228,596	

Table B-3. TEA OPEX Summary for 2000-acre ethanol plant.

2000	WetAcre	Sub.area cost		
		P10	P50	P90
Area	Area code	OpEX ($/acre-y)	OpEX ($/acre-y)	OpEX ($/acre-y)
Innoculum	Area 0100	237	537	537
Production Field	Area 0200	2,571	4,885	8913
Dewatering	Area 0300	334	655	665
EtOH Separation	Area 0400	583	678	840
Biomass Treatment	Area 0500	458	533	533
Nutrients	Area 0600	1084	388	3424
CIP	Area 0700	496	618	1061
Gas Management/CO_2	Area 0800	(1,222)	629	2738
Product Storage Loadout	Area 0900	66	66	66
Wastewater Treatment	Area 1000	319	482	355
Utilities (without Raw material cost)*	Area 1100	128	128	128
Fixed Cost (without Labor for Area 0100-	Area 9000	1107	2288	6140
Sum.		6,161	11,886	25,399

The capital cost estimation is based on the Lang and Guthrieth factor method ("Product and Process Design Principles" by Seider et al 2010), which uses installation factor and individual purchased equipment cost to calculate overall plant cost. The cost estimation is based on the assumption that this is the "nth" plant with 2000-wet acre production field. The sub areas CAPEX and OPEX values for USA deployment are given in Table B-2 and Table B-3.

The operation cost includes raw material, labor cost, PBR replacement cost, property tax and insurance cost. Raw material cost is based on Heat and Material Balance data (ASPEN plus Model, see Figure B-25), and power usage (from a Natural Gas Combined Heating and Power (CHP) Unit, see Figure B-26). CHP will be sized for heating requirement to provide 13,000 kg/hr steam in ethanol separation process and generate average 9 MW electricity (day-night average).

Labor cost is assigned according to labor hour requirements in each area, with reference to Algenol IBR operation experience and input from the relevant project teams.

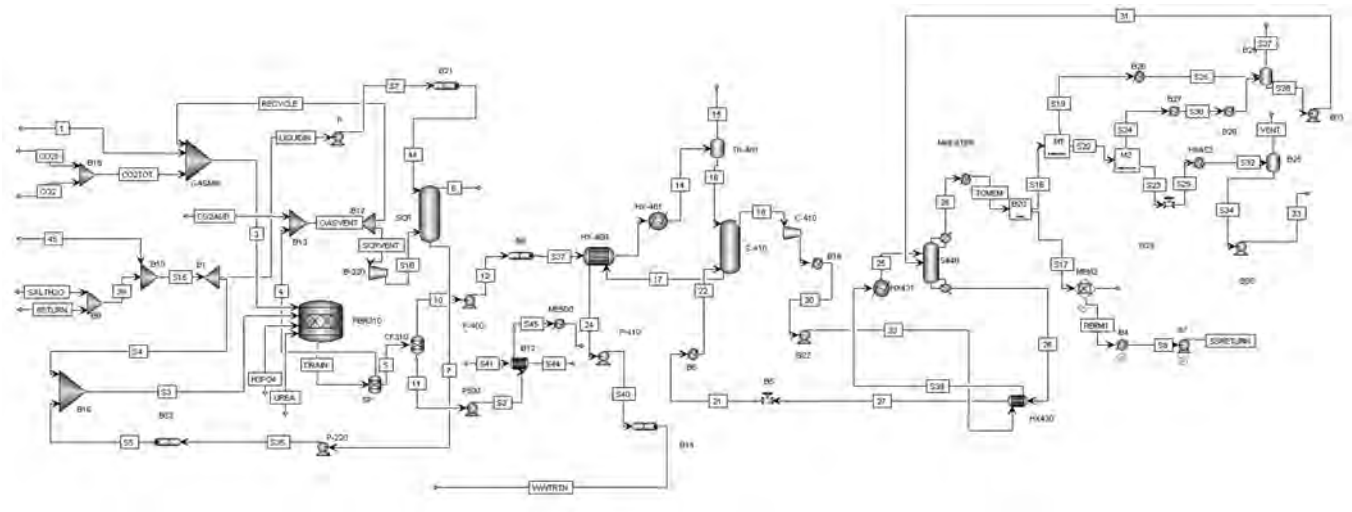


Figure B-25. ASPEN model for Heat and Material Balance Calculation of 2000-acre plant.

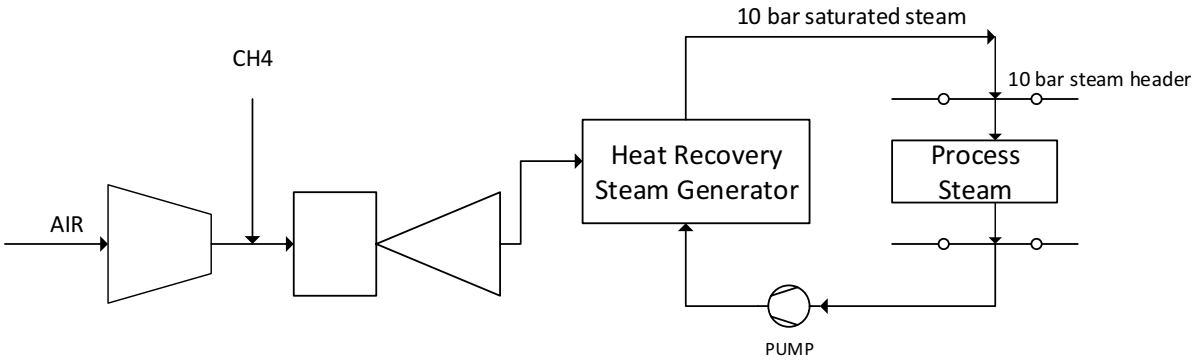


Figure B-26. Process Flow Diagram for CHP unit for ethanol plant.

Based on experience and performance in the latter stages of the IBR project and input from the various project teams, CAPEX and OPEX for P50 targets are calculated. These are given in Figure B-27 and Figure B-28. Areas 200 (Production field), 1100 (Utilities), and 800 (Gas/CO_2 management) are responsible for two-thirds of the CAPEX. On the OPEX side, Areas 200 (Production field) and fixed costs together account for half of total OPEX. In seeking technology improvements and system changes, it is important to keep these factors in mind since cost saving measures in these areas have the highest impact.

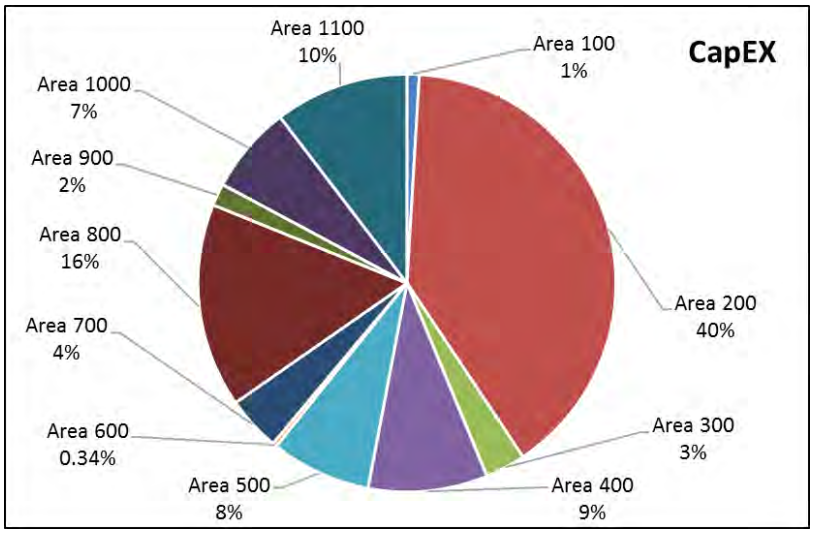


Figure B-27. CAPEX breakdown based on June 2015 data.

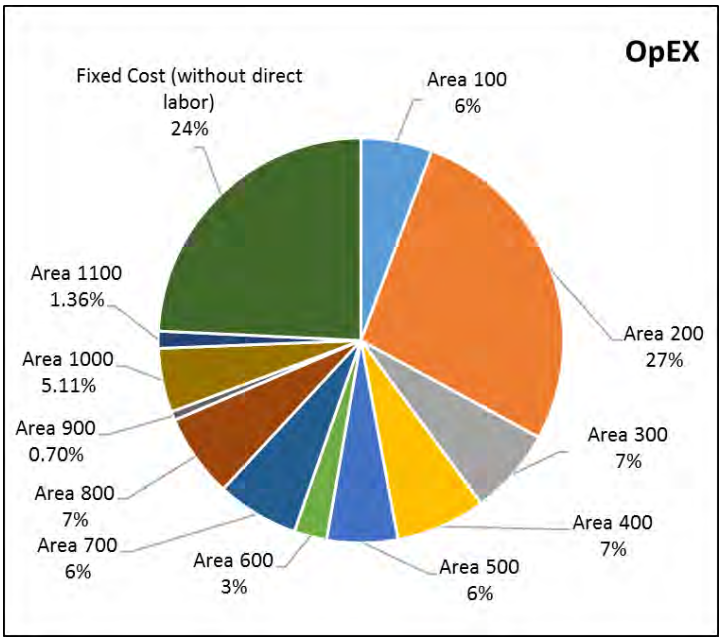


Figure B-28. OPEX breakdown based on June 2015 data.

It should be noted that CO_2 cost in the P50 case is assumed to be 0 $/tonne CO_2, e.g., an assumption that could be consistent with a carbon tax. If CO_2 cost was 25 $/tonne CO_2, it would correspond to about 0.2 $/gal OPEX increase (at 7300 TGOLF). Therefore, low cost CO_2 and high CO_2 utilization efficiency will be important factors for a commercial biorefinery. Algenol has conducted considerable research related to CO_2 capture costs and novel scenarios for integrating CO2 sources with Algenol biorefineries (discussed briefly in LCA section).

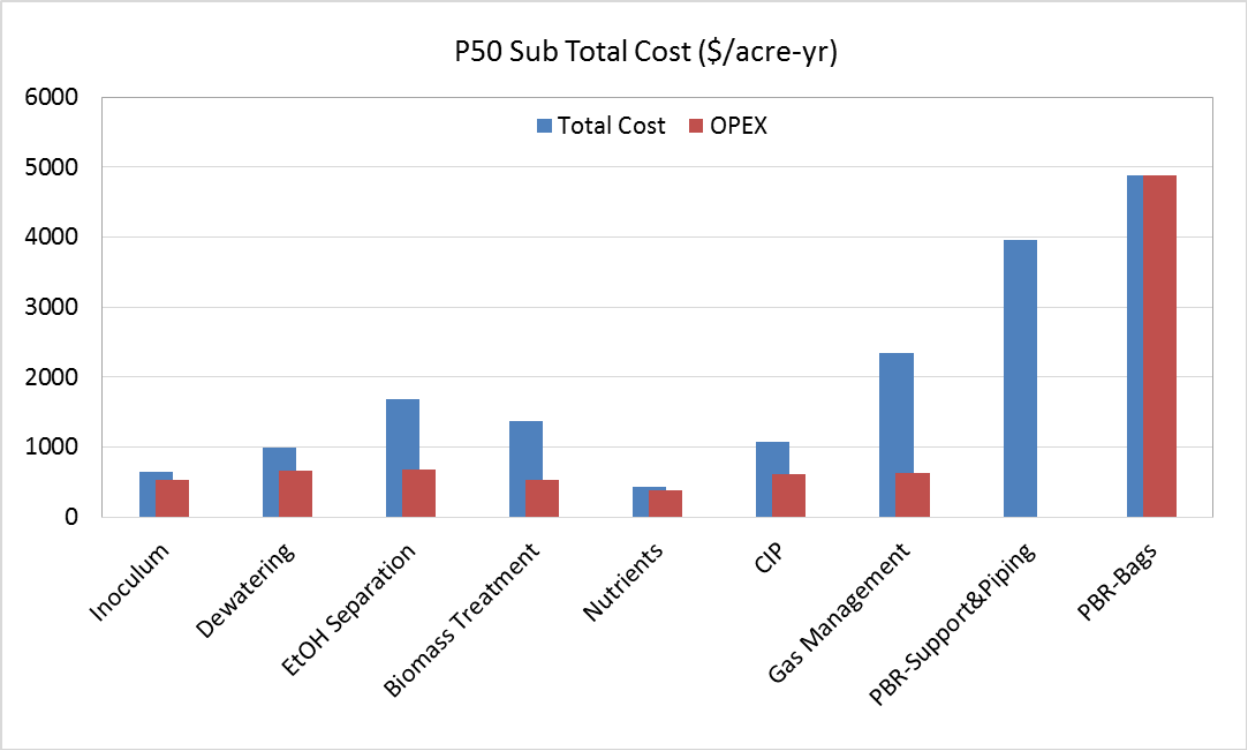


Figure B-29. TEA Cost Breakdown into each AREA for 2000-acre ethanol plant (P50).

In Figure B-29, Total Production cost per wet-acre for the P50 case is calculated from Annualized CAPEX + OPEX; the annualized CAPEX factor is 0.1. PBR bags, PBR support structure and gas management costs are the top three cost items. Current productivity is about 5000 GEPAY, corresponding to a fuel production cost of 8.4 $/gal (OPEX at 4.6 $/gal). With the P50 case of 7300 gal/acre-yr, the total fuel production cost is at 3.5 $/gal (OPEX at 1.6 $/gal). Algenol's long-term target is to reach a 2.7 $/gal total fuel production cost and 1.2 $/gal OPEX; this will require significant cost reduction in the PBR system cost, an increase in ethanol productivity, and low CO_2 cost, i.e., achieving many of the P10 elements of the cost and performance matrix.

Financial modeling for plant economic assessment

The comprehensive TEA plant economic model provides the information with which to prepare financial assessments of plant performance. These assessments are performed in Algenol's Financial Modeling program, which takes the inputs from the TEA and combines them with market assessments of price, the investment environment, and other ongoing economic factors that would drive a plant business model. The Financial Model allows Algenol to project the ethanol plant performance over time by adopting the key assumptions derived from the TEA (capital cost, operating cost and productivity), and adding assumptions regarding product price and related credits, the projected useful life of our PBRs, and financing and debt options (see Figure B-30). Finally, we layer a calculation of inflation and other changing market impacts on these input assumptions, allowing us to model the plant economics over the projected useful life of the plant.

The resulting year over year plant economic projection allows Algenol and investors to assess key performance indicators such as cash flow, EBIT, Internal Rate of Return, Net Present Value of investment flows, and Debt Service Coverage Ratios. In addition, the Financial Model is able to model annual Financial Statement information with Income Statement, Cash

Flow, and Balance Sheet presentations for the life of the plant. The Plant Financial Model is an interactive calculation tool, which permits further sensitivity analyses (including Monte Carlo probability modelling) to help evaluate the relevance of key input assumptions on desired performance indicators over plant life by modifying particular assumptions. The results may be displayed in a tornado graph as illustrated in Figure B-31. This tornado diagram was generated based on P10/P50/P90 assumptions at each major costing area. Two areas stand out: Productivity and PBR cost. The origin of the PBR-related cost assumptions has been discussed earlier in this section. The P90 value for productivity derives from a combination of current outdoor performance (IBR and PBR) with expectations from our modeling efforts. The P50 and P90 productivity values originate from our subject matter experts, our performance data base, and our productivity modeling efforts. (See Productivity Modeling Section for more detailed discussion of the basis for the P10, P50 and P90 values for annualized TGOLF.) The reference point in Figure 15 is the P50 case: Productivity at 7300 TGOLF/6300 GEPAY, and IRR as 5.2%. From the tornado graph, the major economic impacts originate from Productivity, PBR cost, PBR lifetime, and CO_2 cost. Our research efforts have been well aligned with that ordering of importance.

Model Assumptions

CapEx per Acre	Florida
PBR	$ 28,913
Plant	$ 105,159
Total CapEx	$ 134,071
Commercial Project Size (acres)	2,000
Capital Maintenance %	2%
Capital Maintenance - Annual	$ 2,103

Depreciable Life	
PBRs - Start-Up	8
Plant	24
PBRs - Replacement	8
Maintenance CapEx	24

Volume Inputs	Florida
Derated GEPAY	6,300
Green Crude GEPAY	1,000
TGOLF GEPAY	7,300
Productivity Increase (TGOLF %)	0.0%
Productivity Increase Start Year	2020

Price Inputs	Florida
Ethanol ($/gal)	$ 2.73
Production Credit ($/gal)	$ -
Ethanol RIN ($/gal)	$ 0.48
Ethanol CA Carbon Credit	$ 0.25
Green Crude ($/gal)	$ 2.25
Green Crude RIN ($/gal)	$ -
Annual Fuel Price Increase	1.9%
Price Increase Start Year (2nd Yr of Ops)	2018

Operating Expense ($/TGOLF)	Florida
Raw Materials, Nutrients, Utilities	
2k Acres	$ 0.369
Personnel	
2k Acres	$ 0.510
SG&A	
2k Acres	$ 0.226
Total	
2k Acres	$ 1.104
COLA	3.0%
Tax Rate	35%
PBR bags amortization	0.495
OPEX/gallon (USD)	$ 1.60

Financing / Debt Assumptions	
DOE Loan Guarantee	
Loan Term	20
Interest Rate	6.0%
LTV	0%
Facility Fee	
At Term Sheet	0.0%
Issuance Facility Fee	0.0%
DOE Maintenance Fee	-
Credit Subsidy Fee	0.0%
Non-Guarantee	
Loan Term	20
Interest Rate	6%
LTV	80%
Equity	20%
Other Costs	
DSRF	0%
Legal	$ 2,500,000
3rd Party	$ 500,000
Banking Fees	
Guaranteed	-
Non-Guaranteed	2.5%

Figure B-30. Financial Model input assumptions.

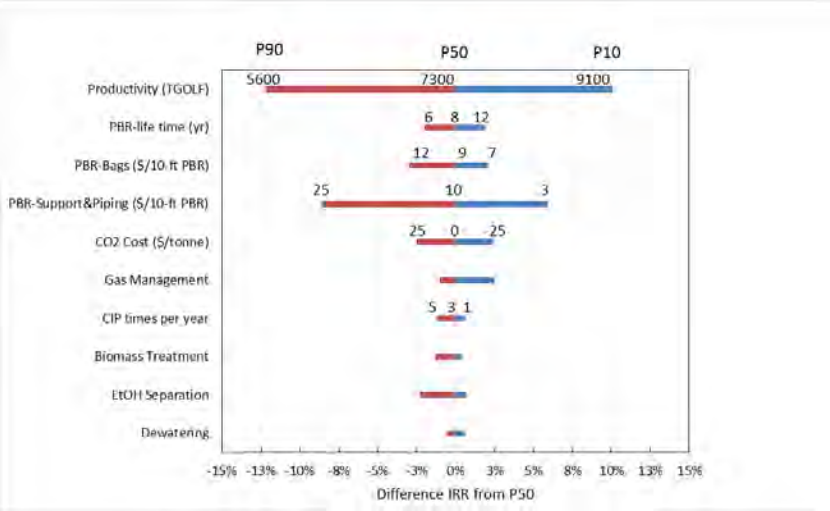


Figure B-31. TEA Sensitivity Analysis for 2000-acre ethanol plant. The IRR for the P50 reference case is 5.2%.

Market conditions are of course the key external driver when evaluating the financial plant model. Algenol reviews reports from both public (EPA and other government agencies) and private organizations (OPEC, etc.) to asses current market prices and the expected trends in pricing over the life of the plant. Furthermore, there are several biofuel credits available to compensate for the high production cost of biofuel. As shown in Figure B-38 three types of biofuel credits can be available for biofuel production: 1) Cellulosic tax credit from the US government, 1.01$/gal tax credit; 2) RINs from US EPA's Renewable Fuel Standard (RFS), the RINs credit is scaled with the energy content of the fuel and greenhouse gas (GHG) reduction (for example, for 60% GHG reduction there could be $1.75 $/gal credit for ethanol; 3) California Low Carbon Fuel Standard (LCFS), 1.34 $/gallon for 100% GHG reduction, although such biofuel would need to be shipped to California and some extra shipping cost will be added.

In Algenol's ethanol plant, we produce 85% of the biofuel as ethanol, and 15% as bio-crude. in the current Algenol Financial Model, the following biofuel credits have been applied only for ethanol but not for bio-crude.

1. Renewable Fuel Credit: Ethanol RIN is 0.48 $/gal
2. California Carbon Credit: Ethanol is 0.25 $/gal

These credit assumptions are rather conservative at this point compared to what is possible from Figure B-32, but there is also offsetting uncertainty in the ethanol price and our assumptions there are optimistic compared to the current market conditions. Overall the system is close to economic viability based on the P50 estimates. Some combination of improved productivity, continued reduction in PBR costs, higher oil prices, and a political climate that essentially guarantees renewable fuel incentives is needed to attract the investments required for deployment of this technology. Algenol continues to work on technical improvements, as well as diversification into higher value products based on some of the technology developments from the current program.

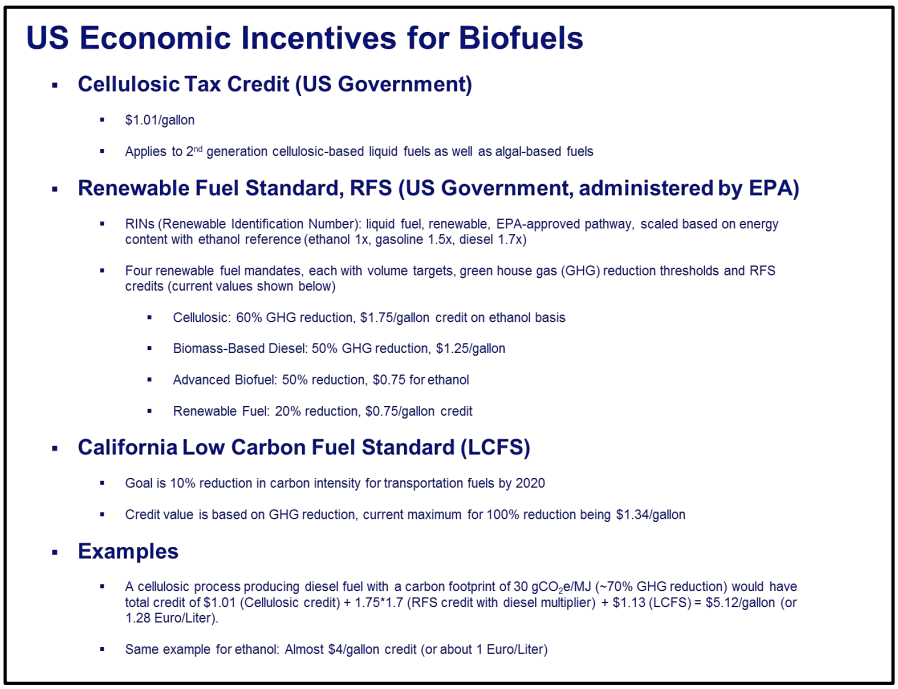


Figure B-32. US economic incentives for biofuels.

Subtask B.6 Life Cycle Analysis (BP-2)

Completed May 2017

The milestone for this area was the submission of a Technical Report that demonstrated, for Algenol's ethanol production process, at least a 20% reduction in greenhouse gas emissions compared to gasoline. The target date for completion was August 31, 2010. That milestone was met in a timely manner and substantially surpassed.

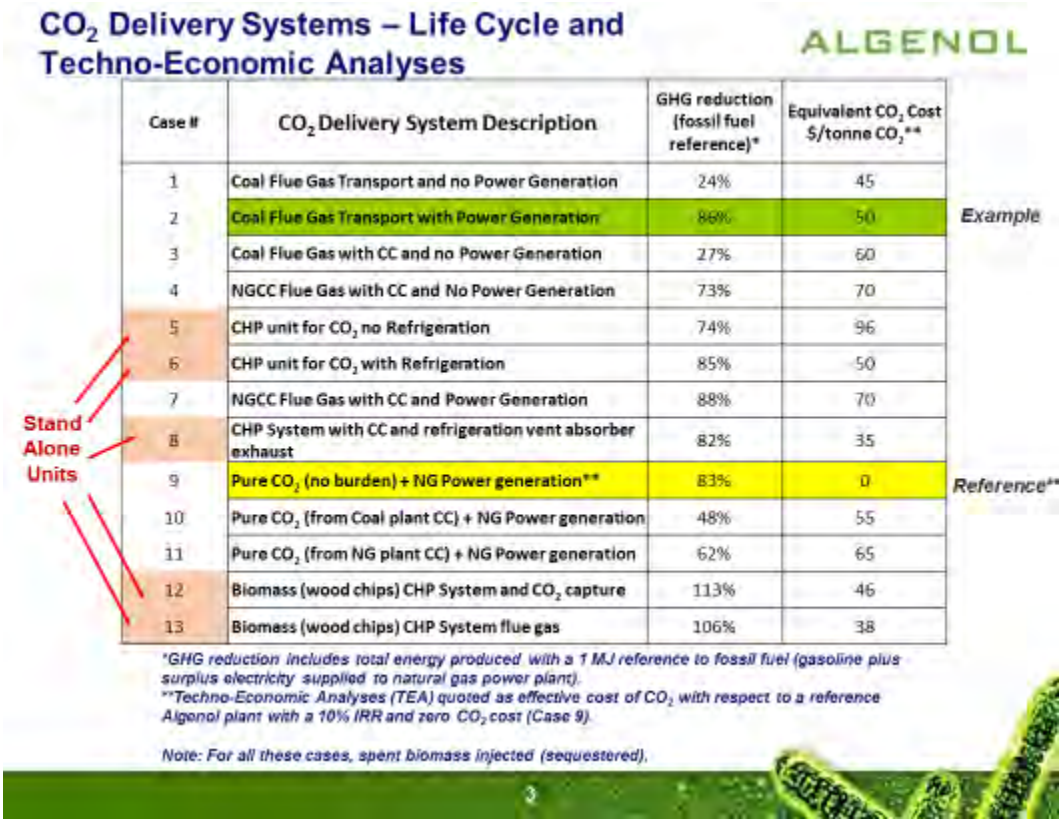
The draft report of collaborative work in this area (Georgia Tech and Algenol, dated June 9, 2009) was submitted with the proposal and suggested that Algenol's process had the potential to perform much better than the stated target of a 20% reduction (same as RFS target for renewable ethanol). That analysis was completed and was submitted to *Environmental Science and Technology* (a highly respected American Chemical Society journal) for publication in March 2010. The paper described calculations for the Algenol Direct to Ethanol system leading to a 67-87% reduction in greenhouse gas (GHG) emissions (expressed as gCO_{2eq}/MJ) in comparison to gasoline. The paper was published in October 2010 (Luo et al, *Envir. Sci. Tech*. **2010**, *44*, 8670-8677). To our knowledge, that paper was the first of its kind in providing a credible, peer-reviewed analysis of an algae-based biofuel production system. The article now has over 100 citations according to Google Scholar.

Based on that work and subsequent improvements and modifications due to changes in the Algenol system, e.g., vertical photobioreactors, Algenol obtained EPA RFS pathway approval, with EPA citing their own work showing a GHG emission reduction of about 70%, which was in good agreement with the Algenol-Georgia Tech work (https://www.epa.gov/renewable-fuel-standard-program/algenol-biofuels-inc-approval).

The LCA work has been kept evergreen throughout the IBR project, and along with the TEA, serves as a research guidance tool. As noted in the TEA section, the TEA data base also serves as input for the LCA work. The LCA work, in turn, serves as input for the Financial Model in that the GHG emission reduction impacts financial incentives for renewable fuels.

Follow-up work, of high importance to the further development of the Algenol process, as well as to biofuels in general, involved the integration of the Algenol plant with anthropogenic CO_2 sources. The focus was on fossil-based power plants as the CO_2 source. A number of scenarios were considered but the basic messages are: 1) Carbon capture and utilization (CCU) can be fully competitive with Carbon Capture and Sequestration (CCS) as long as the GHG emission reduction is at least 75% compared to gasoline and 2) Projected carbon footprint values and estimated production costs for algae-based ethanol are favorable compared to other transportation fuel options, including corn-based ethanol and electricity. A peer reviewed publication (collaboration between Algenol and Georgia Tech) describing this work was published in 2015: R.P. Lively et al, "Anthropogenic CO_2 as a feedstock for the production of algal-based biofuels", *Biofuels, Bioproducts, and Biorefining* **2015**, *9,* 72-80.

The results for 13 CO_2 sourcing scenarios examined by Algenol and Georgia Tech are summarized in Figure B-33, which is is taken from a short presentation given by Algenol at the DOE-sponsored Carbon Capture and Utilization workshop in Orlando, Florida (May 23-24, 2017).



Case #	CO_2 Delivery System Description	GHG reduction (fossil fuel reference)*	Equivalent CO_2 Cost $/tonne CO_2**
1	Coal Flue Gas Transport and no Power Generation	24%	45
2	Coal Flue Gas Transport with Power Generation	86%	50
3	Coal Flue Gas with CC and no Power Generation	27%	60
4	NGCC Flue Gas with CC and No Power Generation	73%	70
5	CHP unit for CO_2 no Refrigeration	74%	96
6	CHP unit for CO_2 with Refrigeration	85%	50
7	NGCC Flue Gas with CC and Power Generation	88%	70
8	CHP System with CC and refrigeration vent absorber exhaust	82%	35
9	Pure CO_2 (no burden) + NG Power generation**	83%	0
10	Pure CO_2 (from Coal plant CC) + NG Power generation	48%	55
11	Pure CO_2 (from NG plant CC) + NG Power generation	62%	65
12	Biomass (wood chips) CHP System and CO_2 capture	113%	46
13	Biomass (wood chips) CHP System flue gas	106%	38

Figure B-33. CO_2 source and delivery systems considered in economic analysis

The first of two planned external publications from this work is in draft form and will discuss Cases 1, 2, 9, 10 and 11. It will update the 2010 work (basically Case 9) to better represent the modern vision of an Algenol plant (e.g., vertical PBRs, improved PBR designs, greater biomass residues, etc.). It will also extend the boundary to include the emission burden associated with the CO_2 capture and delivery system (Cases 1, 2, 10, and 11). As the table indicates, the impact on GHG reduction can be substantial. The economics (preliminary at this stage) are also impacted. Hydrothermal liquefaction of the biomass has been studied, but not systematically included at this stage; instead the biomass residue is sequestered for the results shown in the figure.

The second paper is very high impact in our view. It makes the point that the 5 systems flagged as "stand-alone" in the figure have very attractive GHG reductions, but also, for the most part fully competitive economics based on our preliminary analysis. Those systems all involve generation of sufficient CO_2 on-site to supply the facility, selling the excess power back to the grid. This removes the serious restriction of having to locate an algal-based biorefinery close to an anthropogenic source, e.g., essentially adjacent to a power plant. This constraint would be very restrictive, placing severe limits on the potential impact of biofuels on our fuel supply and on GHG emissions. The stand-alone systems remove that constraint, requiring only a source of fuel for the CO_2 generation (natural gas or wood chips). There is some added complexity, but also an important advantage in that the system is self-contained and not seriously dependent on another business entity.

Subtask B.7 Program management activities for Phase II

Completed December 2014

This is a continuation of Phase 1 program management activities.

Subtask B.8 BP-2 gate review

Completed October 2014

The BP-2 gate criteria were established by the PMT, the IGT and the DOE. The Gate Criteria for BP-2 required: 1) Meeting the BP-2 target level of ethanol production per acre; 2) Meeting the BP-2 target carbon dioxide utilization per gallon of ethanol produced; and 3) Successful completion of the Independent Engineer's performance test report.

Task C – Optimize Operations

Task Objective

Efficiency and cost-cutting were the primary emphases for Task C of this project. The principal activity was the operation of the pilot plant, with a total of 133 batches run in the 15 blocks between January 19, 2015 and December 21, 2016. Completion of a current state analysis and risk analysis, together with implementation of the method of change procedure and root cause analysis allowed for substantial process optimization leading to batch-to-batch stability and improved efficiency. Based on the conditions of stable operations, comprehensive operational checklists and SOPs were finalized. Efforts culminated in the preparation of this final report, which documents the comprehensive technical and operational achievements gained over the project period and provides a thorough economic assessment of viability through Techno-Economic Analysis and Life Cycle Analysis.

Project Activities

Subtask C.1 Revise the production process and procedures to reduce cost and improve efficiency

Completed July 2015

Since the onset of the DOE IBR program, five enabling process and design advances have been critical to the successful development of Direct-to-Ethanol® technology:

1. Identifying a highly productive host strain in AB1 and then successfully developing the molecular tools to enhance metabolism in the organism,

2. Optimizing ethanol metabolism within the host cell to divert over 70% carbon fixed during photosynthesis to ethanol,
3. Developing a vertical flexible-film photobioreactor platform that both improved areal ethanol production and moderated the cultivation environment compared to horizontal PBR systems.
4. Building an internal PBR manufacturing capability that has allowed for rapid prototyping, pilot scale manufacturing capacity, plastic film testing and development, and attention to quality of the proprietary PBR product,
5. Engineering the proprietary Vapor Compression Steam Stripper and primary ethanol separation system that has enabled low energy ethanol separation and purification from a dilute salt water medium.

Process optimization and advances during the program have been discussed in several Task A and B sub-sections. Results at the IBR are presented in Subtask C.2 below. Commercial plant economics are detailed in Subtask B.5, and opportunities for process advances derived from our productivity model are described in Direct-to-Ethanol Production Model section below. Nevertheless, progress and opportunities for process and design optimizations for the most impactful economic drivers are briefly summarized here.

Ethanol production rate – The most significant economic driver is ethanol production, and the process becomes more and more commercial viability with improvements in ethanol production from 5,600 (current) to 7,000 to >9,000 TGOLF. The pathway to improved productivity includes advances in biology, culture operations and engineering. Stain development has significant opportunities to increase overall productivity. As stated, strain advancements in carbon branching to ethanol and genetic stability of the ethanol cassette have resulted in strains capable of both high production rates and maintaining peak rates for several months. Next generation strains will have the ability to remain highly productive in a high ethanol background and have photosaturation and light use characteristics more like the wild type host strain. Baseline photosynthesis and productivity is enhanced in the most advanced strains by optimizing carbon fixation and the Calvin cycle.

Most outdoor ethanol cultures are operated in batch mode where both biomass and ethanol concentrations increase over time. As biomass density increases, overall culture productivity declines as cells acclimate and respiration demand increases. Laboratory experiments suggest overall ethanol production is improved if cultures are operated continuously or semi-continuously. In this case, biomass is maintained at a relatively low concentration that provides high light penetration in the culture and high photosynthesis rates. Continuous operations are s enabled with strains that have highly stable ethanol cassettes. Other operational concepts reduce ethanol lost during cultivation during aeration. Both laboratory and PDU scale experiments demonstrate that most ethanol that would otherwise be lost to vaporization can be retained in the PBR system if gases are recycled.

The photobioreactor design and configuration was determined to be a fundamental driver of ethanol production and is one of Algenol's key enabling technologies. In addition to improving productivity by moving from a horizontal to vertical system, Algenol demonstrated that productivity was largely dependent on the space between PBRs (or more precisely the culture height to space ratio). A series of optimization experiments was conducted to find the optimum spacing that maximized plant profitability by balancing productivity, ethanol concentrations at harvest, and PBR system CAPEX. Further, PBR systems can be engineered to improve light use efficiency by trapping more light and by better scattering incoming light throughout the array (i.e., distributing light more evenly across the vertical face).

Finally, co-product generation can increase overall plant productivity. In Algenol's base biofuels case, the co-product is biocrude made from waste biomass after ethanol separation. Algenol is currently exploring other co-product opportunities as a market entry strategy or that help provide stable plant profit in a potentially volatile fuels market.

CO_2 source and use efficiency – CO_2 is the major process feedstock in ethanol and biofuel production using algae. A common commercial vision is to co-locate an ethanol plant next to a CO_2 point source such as a natural gas or coal fired power plant. We have found several challenges to this approach. First, large expensive pipes are required to transport flue gas from the power plant to the distribution network of the algae plant. This is particularly true in the natural gas case where CO_2 concentrations are relatively low and a large overall air volume is necessary to transport. Pipes can be smaller with coal flue gas but may require expensive scrubbers. In either case, a large parcel of land for the algae farm would have to be available in close proximity to the power plant. In addition, it may be difficult to secure binding take off agreements with a power plant utility company as their business to remain profitable may not align with the requirement to operate a particular flue stack for the lifetime of the algae plant. An attractive alternative, and a significant finding, is that a commercial algae farm would benefit with a stand-alone natural gas (or biomass) power generator located onsite. In this case, the major requirement of the generator would be to supply CO_2 to the field, and excess electricity sold to the local electrical grid. The algae plant would be independent and not tied to a particular partner company or location.

Regardless of the source, CO_2 feedstock is a significant operating cost, and increasing culture carbon use efficiency is an important process optimization. Algenol has demonstrated in laboratory and outdoor gas recycling where CO_2 that would typically bubble through the reactor field and be vented is instead returned back to the system. CO_2 losses are much reduce with gas recycling, and high oxygen tolerances of the host strain AB1 is key to this operation.

PBR system development and cost reduction – With over 2,000 acres of cultivation fields, photobioreactors and associated piping are the largest CAPEX investment in a commercial facility. Thus, PBR engineering is one the biggest opportunities for process optimization and cost reduction. The PBR team continues to work with potential vendors to develop films that are inexpensive at large volumes. New innovations in diffuser material and manufacturing have resulted in cost savings, and new, cheaper and more secure support structure concepts are being tested at the PDU. In this program, with significantly less valves, ports and complexity, the airlift PBR system design was a significant cost savings over previous independent PBR designs. As contamination control processes develop for ethanol production, larger and larger volume systems are contemplated, and these systems continue to reduce valve counts, simplify piping networks, and reduce operation complexity. Nonetheless, film advances have significant promise for overall plant economics with new thin films being tested with much reduced plastic and with continues emphasis of extending PBR longevity from 6 to 10 years.

Subtask C.2 Complete cumulative run hours for the optimization phase of the process

Completed July 2015

Summary of cultivation activities from Phase III: operation and optimization of the Integrated Biorefinery

The final scale for demonstration of commercial ethanol production for this grant was a PBR field of 1.8 acres. Over several months in the latter portion of 2014, a process pavilion, process pad, scale-up pad, and supports for the PBR field were constructed on Algenol's Fort Myers, FL site. A total of 15 PBR blocks made up the PBR field, including 8 blocks of 408 integrated

VIPER 2.3 PBRs, 5 blocks of 408 integrated VIPER 2.3, and 2 blocks of 200 VIPER 3.1 airlift. Block installation was on a rolling basis, with the first block of VIPER 2.3s (Block 1) installed during the final week of December 2014. Water and dye testing was performed for several weeks to monitor fluid and gas movement to ensure that all processes could be performed according to design specifications and the Algenol Recommended Cultivation Standards (ARCS). During the water testing period, checklists and operational logs were made to help improve process flow and minimize errors.

1.8-acre module cultivation officially began with the inoculation of Block 1 on January 19, 2015 following the liquid CIP sterilization process validated at the PDU and 100-block. In February, Block 2 was deployed and inoculated, with additional blocks coming on-line every few days through early May until 13 VIPER 2.3 blocks were in operation.

The batches that were run between January and May brought to light two main challenges that threatened operational stability—algal grazers and early culture stress. Grazers, specifically ciliates, presented the major obstacle to success for the IBR demonstration. Through most of Algenol's cultivation history at the R&D scale, grazers remained a rare and easily contained problem. However, with the progression to large scales with fully integrated (i.e., interconnected) bioreactors and piping, the risk of operational instability from a grazer infestation increased considerably. Ciliates were first documented in 1.8-acre cultures in March of 2015. Investigations during an RCA found grazers in the well water holding tanks upstream of the filters and manufacturing-related imperfections in the filter housings. The filter housing imperfections/leaks resulted in water by-passing the sterile filters, ultimately delivering ciliate-containing well water to the PBR field. With such a high grazer load at the start of the batch, the blocks typically experienced universal culture loss (Figure C-1, left). As a result of these findings, routine CIPs of the well water holding tanks were added to block operations to minimize biological load on the filter; nonetheless, filter housing leaks continued and were challenging to overcome because minimal assistance was provided by the vendor. By late May, approximately 40% of batches had been severely impacted by ciliate grazing. Repairs in early July allowed for the filters to pass static pressure tests, resulting in partial improvements to the block; however, successes were limited and temporary with filtration issues remaining a problem through most of the summer (Figure C-2).

In addition to the universal loss of cultures from ciliate addition at the start of the batch, intermittent loss of cultures to ciliates remaining in the block after CIP was identified as a second failure mode (Figure C-1, right). Three steps were added to the CIP beyond the ARCS recommendations to attempt to mitigate batch-to-batch carryover of ciliates:

- Bubbling was added during liquid fills for mechanical agitation
- The concentrations of bleach and NaOH were increased by five times and the addition time of the caustic solution was changed to evening to increase probability of disinfection and minimize photodegradation of the bleach solution
- VIPER headspace ozone gas treatment was re-implemented to ensure contact of sterilant with all PBR inside surfaces

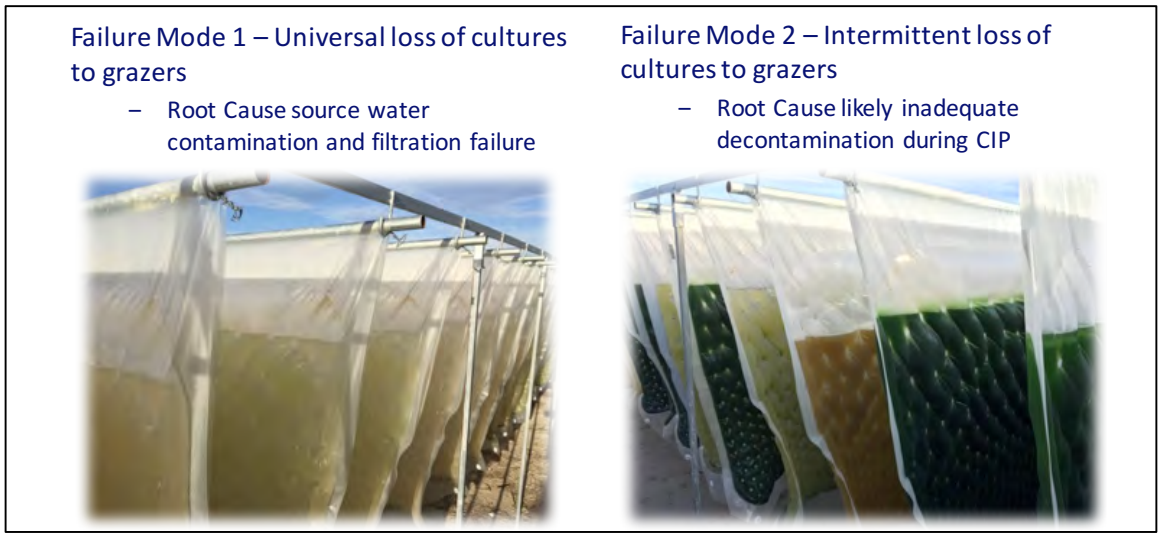


Figure C-1. Photographs showing manifestation of ciliate contamination due to filtration failure (left), where the entire block is lost, or inadequate CIP (right), where culture loss is patchy, likely due to a failure of CIP chemicals to contact every surface in a subset of photobioreactors.

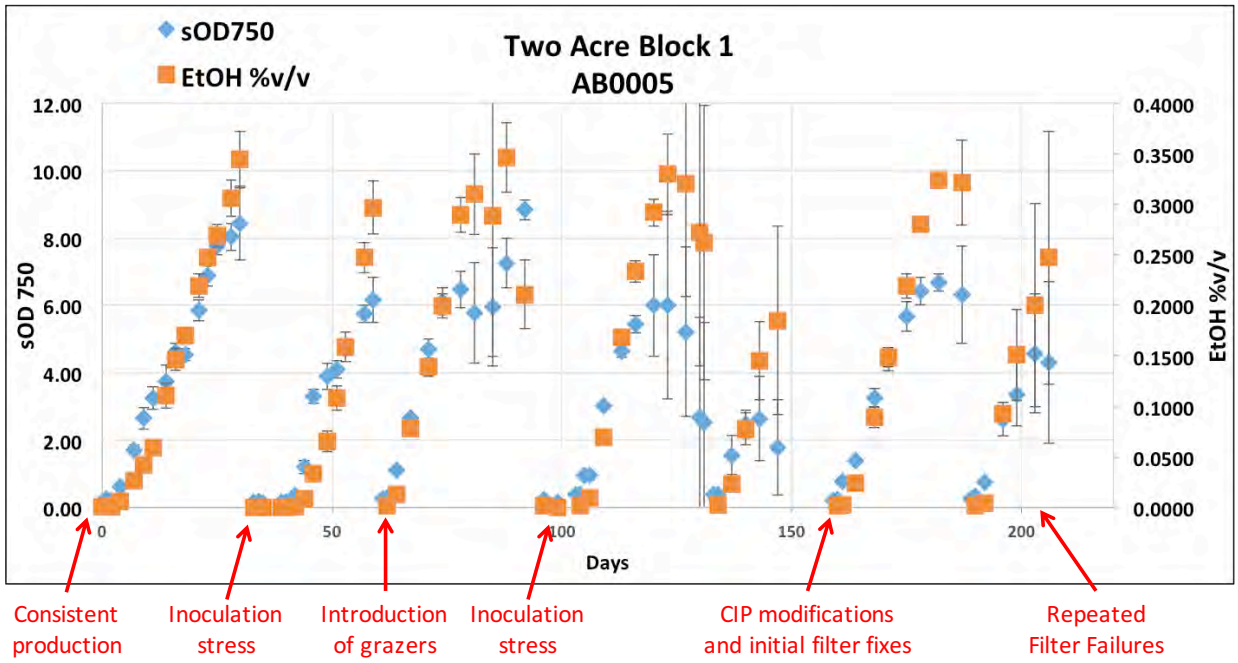


Figure C-2. Biomass (as sOD; left axis) and ethanol concentration (right axis) for batches of AB0005 run at Block 1 at the 1.8-acre IBR demonstration site from January – August, 2015. Challenges from grazers and early culture stress are noted.

Concurrently, batches at the 1.8-acre IBR, particularly those in the first three months of 2015, also suffered inoculation stress (Figure C-2). Based on previous design and operational decisions to minimize inoculum production volume, the operating paradigm when the 1.8-acres began was to inoculate from a relatively high inoculum harvest density (10-15 sOD) to a relatively low production culture starting density (0.1-0.15 sOD). This strategy had showed promise at smaller scales and allowed for dramatically lower inoculum volume requirements. For some batches at the 1.8-acres, however, this strategy resulted in long productivity lags or

even culture death (see examples in Figure C-2). Root cause analysis revealed multiple contributing factors to the lags and culture death, including stress from cold morning temperatures combined with high light intensity (a common weather pattern following the short cold fronts characteristic of Fort Myers during winter), stress from the re-introduction of ozone as a steriliant for grazer remediation, and operational delays leading to block inoculations after sunset. In an attempt to minimize batch losses from culture stress, a short-term decision was made to perform partial-fill inoculations. By inoculating PBRs with the same volume of inoculum, but at a lower total volume, light stress would hopefully be reduced until the culture could grow enough to be topped off to full operational volume a few days later. For the longer-term, overcoming early-stage culture stress became a central research path at the PDU.

The impact of ciliate grazing and inoculation stress was reflected in batch production rates for the VIPER 2.3 blocks (Figure C-2). For harvestable blocks at this time, average productivity was decreased; however, about 38% of block harvests were too contaminated for downstream processing.

Based on these results, a comprehensive plan for attaining operational stability and recovery of ethanol production was developed and implemented in late 2015. To reach this objective, the operational focus was narrowed to the two VIPER 3.2 airlift systems deployed in Blocks 13 and 14, as these blocks had the newest photobioreactors and associated parts and were the least negatively impacted from previous CIP-related ciliate mitigation techniques *(i.e.*, extended CIPs, increased chemical concentrations, re-introduction of ozone). Stability was defined as three consecutive batches without system upsets (design, mechanical, and operational integrity) that produced ethanol at rates comparable to historical performance. An exhaustive current state analysis was performed, risks to stability were identified, and a mitigation plan was developed with the following recommendations:

- Resolve impact of ciliates on batch productivity
  - eliminate leaks by performing extensive leak checks with repair/replacement prior to acid CIP step
  - ensure sterility of cultivation medium by implementing salt water tank CIPs, filter housing integrity testing, and contaminant monitoring
  - implement ciliate control strategies to mitigate batch-to-batch ciliate carryover including airlift circulation of CIP chemicals for increased chemical contact and utilize ciliate control chemicals if necessary
- Minimize negative impacts of early stage culture stress and late stage ethanol consumption
  - implement low optical density inoculum harvest to increase hardiness of culture
  - improve CO_2 control with frequent pH monitoring
  - implement chemical residue measurements during final rinse step with pass/fail criteria for inoculation
- Improve/simplify operational checklists and maintenance records to maintain mechanical and operational integrity
  - implement “management of change (MOC)” procedure for recommended process/design changes

The first two batches in the VIPER 3.2 airlift blocks (13.1 and 14.1) were commissioning batches before the recovery plan was implemented. Both batches produced ethanol; however, 13.1 failed its MACL and 14.1 exhibited substantial ethanol consumption, highlighting the need for an improved CIP process and monitoring, greater control of CO_2, and improved system integrity. Changes were implemented to CO_2 delivery and inoculation methods over the next few batches to align airlift operations with updated ARCS standards to address these issues. In

addition, exhaustive leak checks of all tubes, PBRs, and piping in the block were implemented at each step of the CIP operation. Examples of successes based on these processes are documented below.

CIP monitoring – The failure of achieving the MACL target for batch 13.1 prompted high resolution monitoring of CIP parameters for the next inoculated batch (13.2). Batch 13.2 met the MACL target and total colony counts were reduced by 10^6 by the CIP process; however, this batch also suffered a very long growth delay with visible culture clumping. RCA indicated that residual chemicals were the root cause of the delay and clumping. Processes were implemented to improve the final rinse and monitor residual chemical levels for the next inoculation (14.2). Extra rinses were added until residual oxidants from the CIP were below detection limits. This extra monitoring and rinsing resulted in a considerably shorter growth lag during the next inoculation (Figure C-3).

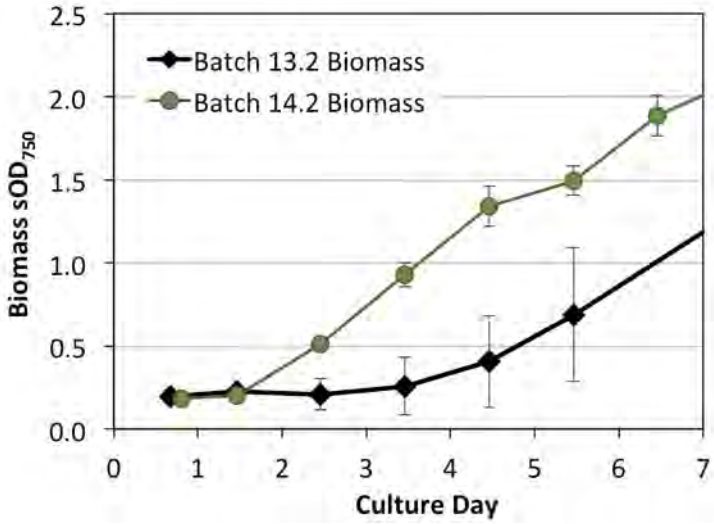


Figure C-3. Biomass (as sOD_{750}) during batches 13.2 and 14.2 (before and after implementation CIP residual chemical measurements and extra rinsing.

CO_2 delivery – Operations were changed to biomass based CO_2 delivery set-points to more tightly control pH, particularly during early batch cultivation when the culture was most sensitive to system upsets (Figure C-*4*).

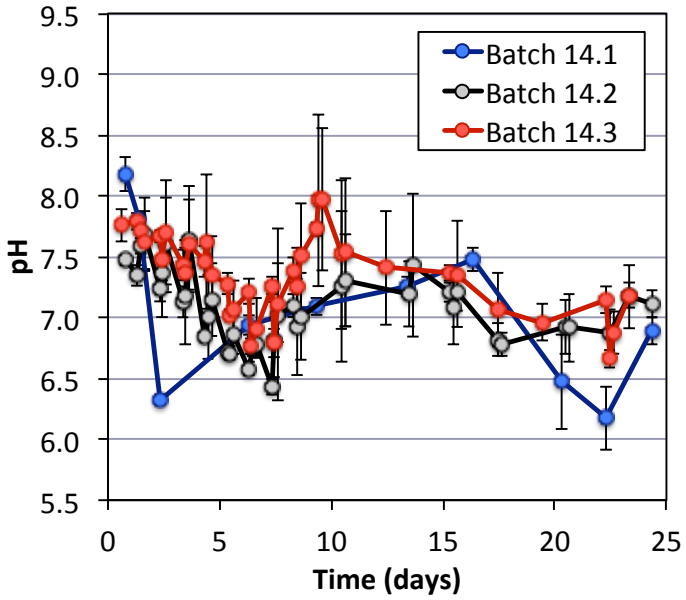


Figure C-4. Improvements in culture pH control from batches 14.1 to 14.3

Leak checks and system integrity – System breaches were identified during the current state analysis and risk analysis as potential contamination sources of bacteria and/or grazers. During leak checks, the spin barbs, tube/barb interfaces, mandrel welds, and PBRs themselves were all identified as common sources for leaks. Thorough leak checks were implemented in January 2016 as part of an MOC to improve system integrity. In addition, crimp-style hose clamps were added to all tube/barb interfaces and the air-in headers were replaced with new UV stable head-welded barbs. By implementing the thorough leak check prior to the final sanitation steps of the CIP, leaks can be fixed to minimize contamination in the block and inform design (Figure C-5).

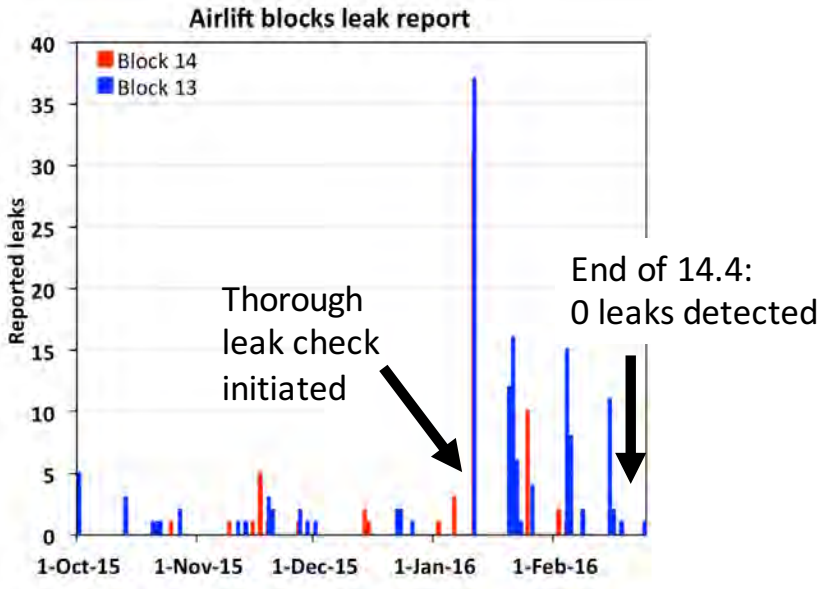


Figure C-5. Reported leaks for blocks 13 (blue) and 14 (red) increased dramatically once thorough leak checks were implemented, largely from leaks at tube/barb connections and spin barb leaks that were only apparent upon barb manipulation. Repair of leaks during CIP prior to inoculation allowed

for system integrity over a full batch, as no leaks were found for an extensive leak check performed at the end of batch 14.4.

Stable operation at the IBR

Ciliate detection, persistence, and recovery in 1.8-acre airlift blocks – Ciliates appeared in the 1.8-acre airlift blocks 13 and 14 during the third batch for each block. RCA revealed the root cause of the ciliate appearance was an operational error. On day 8 of Batch 13.3 (day 4 of Batch 14.3) the blowers were turned off to repair a leak but the CO_2 side-stream sampler inadvertently remained on. This caused a temporary vacuum in the exhaust system that allowed culture to siphon into the shared block headers. Ciliates likely entered from two points—from the back-flush of culture from the exhaust header during recovery from the vacuum and from spin-barb leak points during the vacuum state. To ensure this problem did not recur, the sidestream sampler was relocated and as discussed above, tube/barb leaks were mitigated with crimp clamps. A full CIP was performed to determine if the airlift design allowed for complete removal of ciliates from the system. Unfortunately, ciliates were detected on day 14 in the subsequent batch (14.4), confirming that ciliates could not be removed by the CIP process alone. A secondary ciliate control was needed that could control grazer populations during cultivation without harming cyanobacterial cells or limiting ethanol production. Quinine sulfate (QS) was chosen as it was a known antiprotozoal agent and had already proved effective in the literature at targeting ciliate populations in outdoor algal cultures. First, small-scale QS toxicity tests were performed to determine QS concentrations needed to kill 1.8-acre ciliates without reducing ethanol productivity. Following this recommendation, a QS delivery system was designed to meet dose and delivery rate requirements. The quinine strategy was successful in controlling grazing from ciliates and allowed for recovery to stable block operations for 3 consecutive batches in block 13 before the block was decommissioned and 6 consecutive batches in block 14 before the block was decommissioned (Figure C-6).

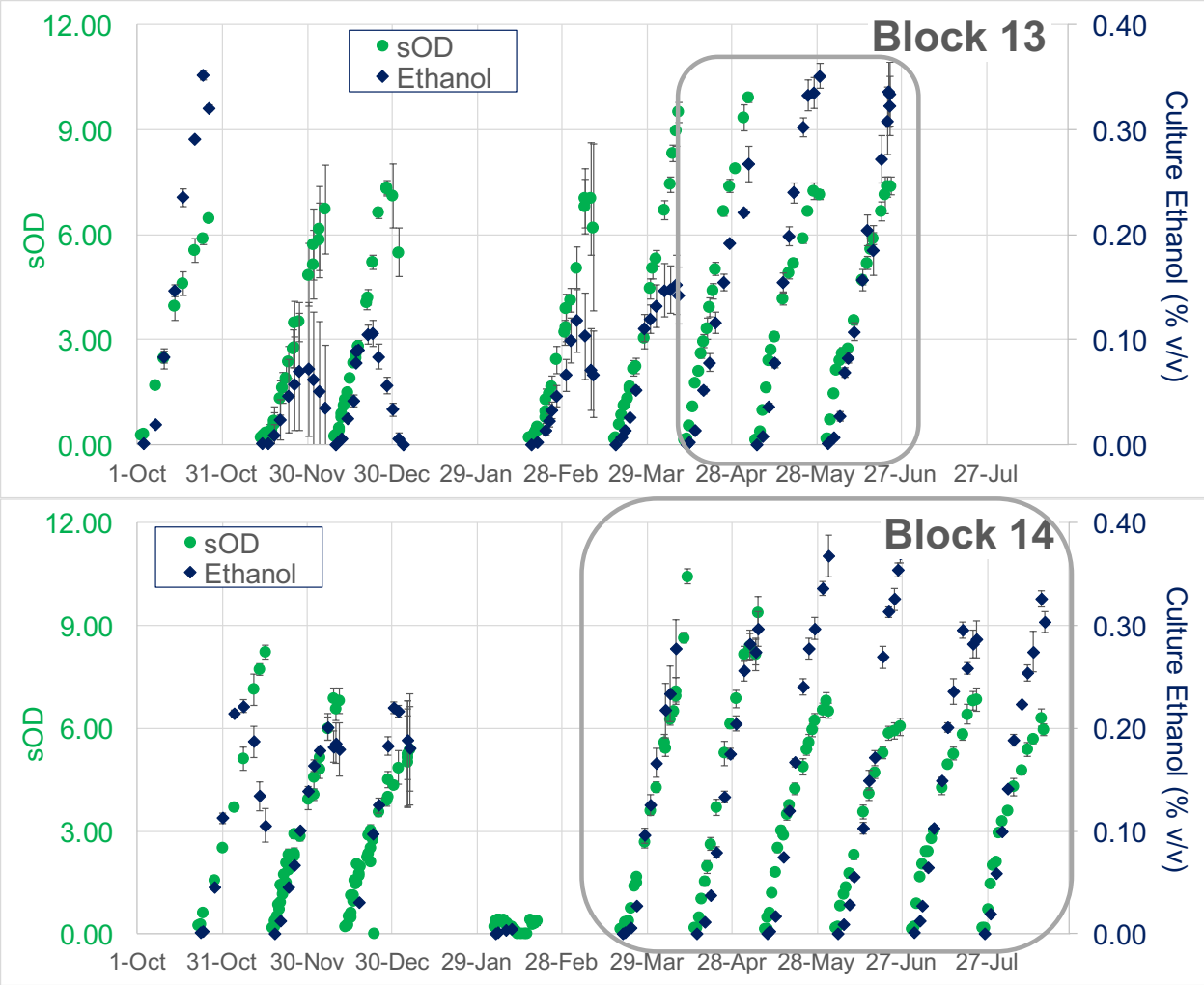


Figure C-6. Biomass (as sOD; left axes; green markers) and culture ethanol concentrations (right axes; blue markers) for all batches run at 1.8-acre airlift blocks 13 (top) and 14 (bottom). Ciliate grazing, cold temperature stress, and ethanol consumption affected early batches. However, after implementation of the quinine sulfate delivery system, batch-to-batch stability was demonstrated for batches 13.6 through 13.8 and 14.5 through 14.10 (highlighted in gray rectangles).

Summary of batch operation – The following summary presents the batch cultivation and operational parameters in effect for the block 13 and 14 stable operation period. These parameters represent Algenol's recommended cultivation standards at the end of the DOE IBR grant period and would effectively be the starting point for future ethanol production optimization (Table C-1).

Table C-1. Algenol recommended cultivation parameters.

Parameter	Recommendation	Comments
Strain	AB0005	n/a
Cultivation Platform	VIPER 3.2 Airlift	Integrated block of 4 airlift systems, each airlift with 50 VIPER 3.2 PBRs plus associated liquid and gas header systems

Parameter	Recommendation	Comments
Salt water sterilization	Ultrafiltration to 0.2 µm	CIP of filter housings and filter integrity testing before every batch
System/material sterilization	Robust CIP	CIP with caustic step and acid step; measurements of total residual oxidants to aid in complete rinsing
Inoculum production vessel	120-L COBRA PBR	Cultures grown repressed in 120 L COBRA PBRs
Batch length	23 days	n/a
Cultivation medium	Phosphate-free ABG-11 amended with urea.	Nutrient stocks ultrafiltered and added through co-flow with sterilized salt water.
Phosphorus feeding	Daily-dose	Seasonally-adjusted concentrations,
Ciliate control	35 ppm quinine sulfate target	Added to block as 8000ppm stock to final concentration of 35ppm when ciliates first detected. Daily addition based on known degradation rates

Ethanol production at Lab, PDU and IBR scales

Under the conditions outlined in Table C-1, we were able to run 9 total consecutive batches at the pilot plant (batches 13.6 through 14.10) to demonstrate stable operation with consistent ethanol production averaging about 3100 GEPAY. Controlling ciliate grazing was a major achievement to help reach the goal of stability; however, rates remain lower than anticipated based on lab results, some PDU results, and annual models. As the focus was shifted off of stability, productivity gaps came in to focus. To determine the factors causing lower than expected production rates, an RCA was performed in conjunction with the deployment of PDU and LvPBR controls for 1.8-acre inoculations. Further, a comprehensive analysis of productivity across various scales was conducted based on the extensive indoor and outdoor experimental datasets in the historical database. The RCA investigated parameters such as inoculum harvest density, UV exposure, CO_2 delivery method/potential carbon limitation, nutrient preparation methods and nutrient limitation, well water quality, impacts of delayed induction, residual CIP chemicals, quinine sulfate addition, and P-delivery methods. Comparisons of outdoor PDU experiments with indoor and outdoor controls for the 1.8 acre batches were useful for eliminating many potential root causes, including water quality, delayed induction, nutrient preparation and limitation, quinine sulfate addition, P-delivery methods, UV exposure, etc. For example, from inoculation of indoor LvPBRs, PDU VIPER 2.3s, PDU airlifts, and block 14 simultaneously with the same source culture and nutrient mixes originating from the pilot plant, inoculum, nutrients, P-delivery methods, and quinine sulfate additions were eliminated as root causes of the low productivity observed in the outdoor platforms. The indoor cultivation of the pilot plant inoculum grown in medium with pilot plant nutrients with quinine delivery showed a productivity of 5569 GEPAY (5625 annualized), which corresponds with predictions from our annual model average productivity of 5600 GEPAY. In contrast, the same inoculum/nutrients cultivated in previously used (and CIP-cleaned) PDU and pilot plant PBR systems had final batch productivities ranging from 2090-3825 GEPAY. Of further note is the similarity between early production rates of the indoor LvPBR control for batch 14.6 and a contemporaneous PDU experiment for the strain advancement team. Mid-batch production rates were similar for all

cultivations, confirming inoculum quality. These comparisons imply that ethanol production lags and ethanol consumption towards the end of the batch (obvious for the PDU airlifts and IBR batch 14.6) are likely the main causes of lower productivity at the pilot plant and specific PDU batches.

As program activities came to a close, productivities across scales for batches without special cause variations (ethanol consumption, growth lags) typically reached or exceeded 5000 TGOLF (Figure C-7), similar to indoor cultivations and demonstrating the capability of the technology to reach scale.

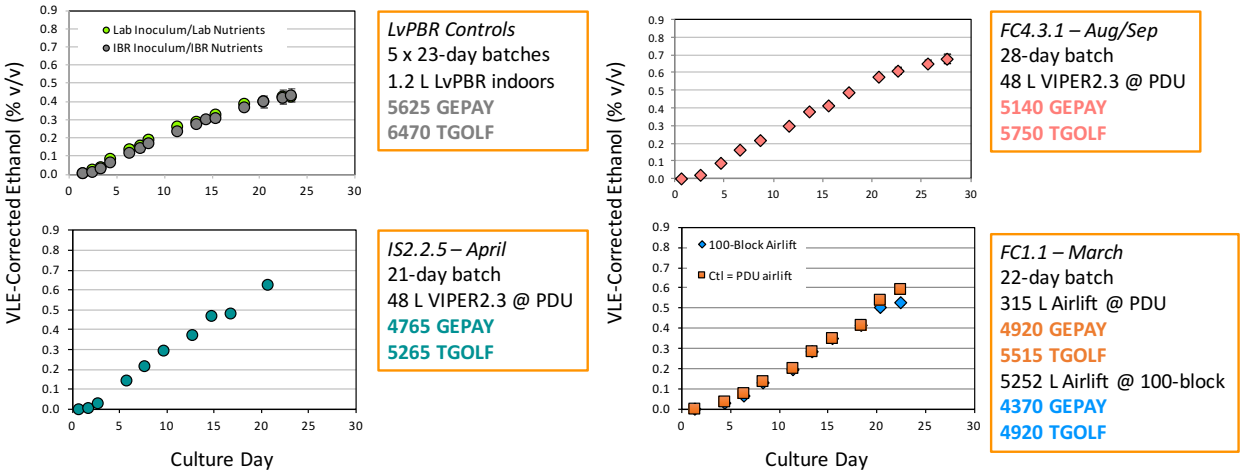


Figure C-7. VLE-corrected ethanol and annualized productivity rates for experiments across varying scales. The selected experiments were not impacted by special cause variations such as ethanol consumption or inoculation lags, and represent base case productivities for routine batches across scales.

Subtask C.3 Complete comprehensive technical, operating, and financial reports acceptable to DOE

Completed May 2017

Subtask C.4 Submit final technical report

Completed May 2017

Direct-to-Ethanol® Production Model

Production Model Introduction

This section provides a summary of the development of a phenomenological model aimed at understanding the productivities of ethanologenic cyanobacteria cultures. The modeling efforts target understanding the ethanol and biomass productivities in small laboratory PBRs as well as large outdoor PBRs, including biorefinery level plants. Models are developed that have specialized applicability to horizontal PBRs, vertical PBRs, and highly mixed PBRs. Illustrative applications of the model to current ethanologenic systems are described. The model provides a good representation of the ethanol and biomass productivities with parameters for quantum yield, ethanol branching ratio, and respiration rate that are reasonable, and numerically consistent with independently obtained estimates. The model provides an annualization process in which seasonally acquired data can be projected to an annual yield based on historical climate data.

Beginning in early 2011, photobiology became a focus area in a collaborative effort that involved the engineering, physiology, biology, and aquaculture groups at Algenol. The primary goal of the photobiology effort has been the generation of sufficient understanding of ethanol and biomass productivities to:

- reconcile small-scale laboratory results with large-scale outdoor results
- quantify incentives for culture management strategies
- provide photosynthetic parameters as a monitor of culture health
- develop predictive capabilities for productivity given local climate inputs
- develop a methodology ("annualization") to allow prediction of annual results from seasonal measurement
- identify phenomena placing limits on outdoor culture productivities
- provide guidance, and quantitative incentives, for research efforts aimed at improved productivities

Algenol made substantial progress in all of these areas, as will be described below and elsewhere in this project report. We will provide here a description of our phenomenological modeling approach and apply that model to productivity results from long-duration indoor and outdoor cultures. The intent is not a detailed mechanistic study, but a working model that deals with the main features of the system in a phenomenological manner in order to provide research guidance for system improvements. The focus is on developing the simplest model that can help to provide a functional or practical understanding of the observed productivities at all levels (laboratory to large scale outdoors) and the changes in productivity that occur as a culture ages. The model has thus far been quite successful with respect to the broad goals of the effort. The focus is on irradiance effects, since temperature changes within a normal operating window (say 20 °C to 40 °C) seem to have minimal effects, at least under our light conditions.

Photosynthetic Model for the Dependence of Areal Productivity on Irradiance

Horizontal PBRs in low mixing (static) limit

Mixing rates are low enough in our systems to consider the system static. In other words, the mixing is not sufficient to move the cells in-and-out of the illumination volume on the time scale of photosynthesis. As will be seen shortly, we can approach the fast mixing limits ("light integration") in the lab, and will model that, but such mixing would be prohibitively expensive for a commercial operation. The model for areal productivity (P_a) requires a description of the dependence on irradiance as the primary variable. We will define P_a for production of fixed carbon (C_{fix}) and address the ethanol production later. We take the simplest, but most commonly employed model for the dependence of P_a on irradiance, E. This approach, in its simplest form, was originally suggested to Algenol by Professor Ed Laws, a LSU professor and Algenol consultant. Algenol has expanded the original suggestion substantially to include the production of both ethanol and biomass, ethanol "quenching" effects, acclimation to lowered irradiance as the culture matures, mixing rates (at least in the extreme limits), and respiration. We have approximate treatments for temperature effects, but have not completely sorted out acclimation effects due to temperature. To this end, temperature effects are largely ignored.

Assume that PAR (Photosynthetically Active Radiation, 400 – 700 nm) irradiance (E) decays exponentially in the culture according to the equation

$$E = E_0 e^{-kZ} \qquad (1)$$

where k is the extinction coefficient and z is the distance into the culture. As a practical matter, k is determined as an average of the absorption spectrum over the PAR range (400 to 700 nm). We have shown Equation 1 to be valid via detailed studies of the adherence to Beer's Law under a variety of conditions. We assume further that the photosynthetic rate per unit volume in the culture at a point z below the surface is described by the hyperbolic equation (basically a Michaelis-Menten approach):

$$P_V = P_m\, E/(E_k + E) \qquad (2)$$

where P_v is the light-saturated volumetric photosynthetic rate (mol fixed carbon/m^3-s) and Ek is the half- saturation constant which describes photosaturation (µmol photons/m^2-s). This formula can be adapted to include photoinhibition effects, but we find that under most lab and outdoor conditions now employed at Algenol, those effects are small. This commonly used form is based on the Michaelis-Menten kinetic formulation and is easily derivable from the 1967 paper by Caperon [J. Caperon, "Population Growth in Micro-Organisms Limited by Food Supply", *Ecology* **48**, 715 (1967)]. The areal productivity (mol C/m^2-s) in a culture of depth D is derived by integrating Equation (2) over the depth (D) of the culture:

$$P_a = \int_0^D P_m\, E_0\, e^{-kz}/(E_k + E_0\, e^{-kz})\, dz = (P_m/k)\, \ln((E_k + E_0)/(E_k + E_0\, e^{-kD})) \qquad (3)$$

where the z integration is performed over the limits 0 to D. In the limit where kD approaches zero, this expression reduces to:

$$P_a = P_m\, E_0 D/(E_k + E_0) \qquad (4)$$

The amount of light absorbed in a very shallow culture is kDE_0. Hence the ratio of areal production to light absorbed in a very shallow culture is:

$$P_a/kDE_0 = Pm/(k\,(E_k + E_0) \qquad (5)$$

Now taking the limit as E_0 approaches zero, we find that the limiting quantum yield (α, reciprocal of the minimum quantum requirement) is

$$\alpha = P_m/k\, E_k \qquad (6)$$

which, when multiplied by E, corresponds to the limiting areal ethanol production rate (mol ethanol/m^2- sec) at low light levels.

For our most common case of a totally absorbing culture (D is roughly 10-20 times 1/k in in most of our experimental cultures), e-kD is very close to zero, and essentially all the light is absorbed. In that case, we have

$$P_a = \alpha\, E_k\, \ln(1 + E_0/E_k) \qquad (7)$$

which takes the proper form ($P_a = \alpha\, E_0$, full light integration) as E_0 approaches zero. If the culture is optically thin, Equation (3) can be used. Equation (7) is the final form that we will often use for most of our analyses of horizontal, dense, static cultures. The complete equation is used when appropriate.

This completes the first pass, or simplest version model. α has an absolute maximum of 0.125 or Cfix/photon for carbon fixation (8 photons per fixed carbon), and 0.042 (1/24) for ethanol production (3 fixed carbons per ethanol). E_k values obtained in our labs are typically in the 50-200 µmol photons/m^2-s range based on irradiance-induced based on O_2 evolution (PE experiments).

Application to vertical PBRs in the static limit

The above discussion applies to horizontal PBRs. For vertical PBRs deployed as arrays of vertical "flat panel" PBRs spaced appropriately to minimize internal shading effects, the illuminated surface area is larger, potentially much larger, than the footprint area of the system. The illuminated-to-footprint area ratio is designated as F, the light dilution factor. For the current Algenol design F is ~10. In reality, the light dilution is non-uniform over the PBR surface, and varies as well during the course of the day (and seasonally). [Algenol has developed models for that light distribution for arbitrary PBR orientation/spacing and any position on the globe.] For our purposes here, it is useful to consider the uniform case. Though we can follow the same process as above, it is easy to relate this situation to the horizontal case. Equation (7), modified for a new diluted irradiance of E_0/F, becomes:

$$P_a = \alpha E_k \ln(1 + E_0/F\, E_k) \qquad (9)$$

which gives the areal production rate per area of illuminated surface. To go back to a more relevant reference of the device footprint area, we must multiply by F,

$$P_a = F\alpha E_k \ln(1 + E_0/F\, E_k) \qquad (10)$$

Equation 10, is the limit of low irradiance or large F E_k, reduces to $P_a = \alpha E_0$ (full light integration), as does Equation (7) under corresponding limits. That shows that when photosaturation effects are absent (which those limits define), there is no advantage for a vertical system. When photosaturation is important (high irradiance or low E_k), the areal productivity in a vertical system is higher, approaching an F-fold increase at the extremes. Those extremes are, however, quite high and for realistic values of E (700 μmol/m^2-s), E_k (50-200 μmol/m^2-s), and F = 10, the enhancement factor for vertical over horizontal PBRs is in the 2-4 range. A more complete application of the model would involve measurement or calculation of the light distribution across the panel surface and explicit calculation of the production rate. That has been done and shows this uniform approach to be a useful, though non-exact, approximation.

Application in the limit of fast mixing (Crison PBRs)

In a laboratory setting, Algenol has employed "Crison" PBRs which are set up to operate at high mixing rates, employing stirring up to 700 rpm. Studies of productivity vs mixing rate show that the "fast mixing limit" is reached at about 400 rpm, producing about a 3 fold increase in yield compared to the static limit. In the limit of fast mixing, where all cells see the same the irradiance, averaged irradiance over the culture column of depth D (horizontal configuration) can be expressed as

$$E_{avg} = \frac{E_0(1-e^{-kD})}{kD} \qquad (11)$$

When this averaged irradiance is applied to Equation (2), the volumetric productivity becomes

$$P_v = P_m E_{avg}/(E_k + E_{avg}) = P_m E_0(1- e^{-kD})/(E_k kD + E_0(1- e^{-kD})) \qquad (12)$$

Given the relationships of $P_m = \alpha E_k k$ from Equation (6) and $P_a = P_v D$, the areal productivity with finite irradiance absorbance becomes

$$P_a = \alpha E_k kD\, E_0(1- e^{-kD})/(E_k kD + E_0(1- e^{-kD})) \qquad (13)$$

In the strong absorption limit where kD>>1, e-kD is very close to zero, $E_{avg} = E_0/kD$ and Equation (1) reduces to

$$P_a = \alpha E_k kD\, E_0/(E_0 + E_k kD) \qquad (14)$$

This condition yields a significant increase in productivity over the low mixing or "static" case described by Equation (7), about a factor of three with rational choice of parameters. This equation is thought to apply to Crison PBRs at high mixing rates (e.g., 400 rpm) where kD is generally much greater than 1. For vertical PBRs with a light dilution factor of F (see above), the denominator in Equation 14 would be replaced by $E_0 + F\ E_k\ kD$, for some further gain in light dilution, but under most circumstances only a small gain in productivity.

Sample calculations of areal ethanol productivity

To illustrate, some sample calculations are shown in Figure Mod-1 where ethanol areal productivity (P_e) is plotted versus irradiance. The reference area for the areal calculation is always the footprint area unless stated otherwise. All calculations use $\alpha = 0.09$, a common "good" result for the limiting quantum yield of carbon fixation. The ethanol (3 fixed carbons) quantum yield (αe) is $\alpha\ \varphi\ /3$, where φ is the branching ratio or the ethanol production rate divided by the rates for all possible dispositions of fixed carbon (biomass, dissolved organic carbon, ethanol, and respiration). Full integration ($P_e = \alpha_e\ E_0$) describes the yield limit where all absorbed light is utilized to produce ethanol, the only constraint being the branching ratio (φ =50%). This limit is achievable only at very low light levels ($E_0 << E_k$). For the fast or "infinite" mixing limit, mixing is fast enough so that all cells see the same light level which is E_0/kD. Crison PBRs operated at 400 rpm or above are thought to be at or near this fast mixing limit; kD, which is about 1.5 x OD750, ranges from a small starting value of 3-5 after inoculation to as much as 30-40 after several weeks of operation at typical irradiance levels (about 400 µmol/m2-sec). We have used kD=10 for the sample calculations. The E_k value is taken as 100 µmol photons/m2-sec for all calculations, a bit of an overestimation for the Crison, since the average light is generally substantially lower than that for the horizontal or vertical PBRs. The horizontal PBR results show the largest photosaturation effects, and under average annual Florida irradiance levels (680 µmol photons/m2-sec), the areal productivity is about 3000 gal/acre-yr. This is somewhat higher than our best results for ABCC171 (an early ethanologenic derivative of our base AB1 strain) in a horizontal system where our data analysis yielded φ =40%. The vertical results shown in the figure (about 7500 gal/acre-yr) are also higher than our best results thus far, even with higher branching ratios. As we will see shortly, the issue is primarily decreasing photosynthetic capacity (as exemplified by changes in E_k and P_{max}) as the cultures grow and densify.

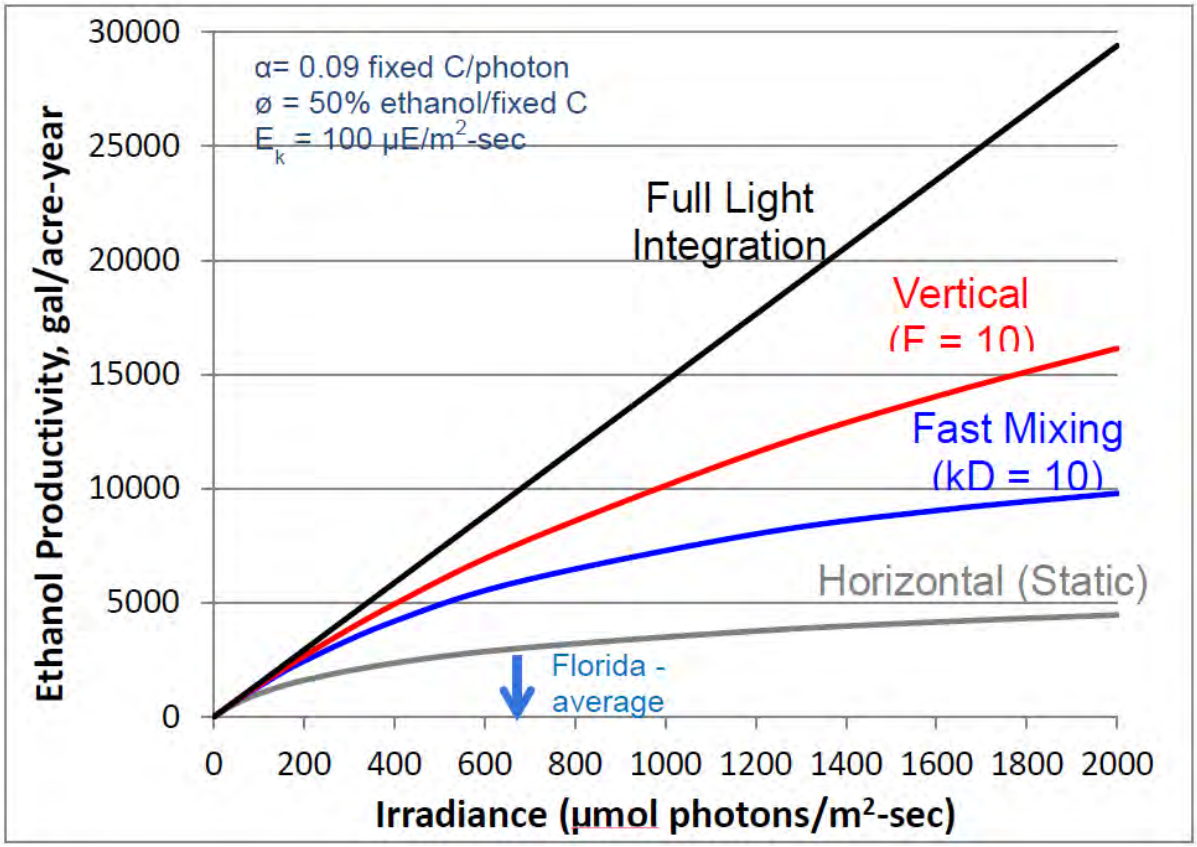


Figure Mod-1. Results from productivity model for various PBR configurations. Results are for ethanol productivity, where the quantum yield for carbon fixation (α) is taken as 0.09 mol C_{fix} /mol photons and the ethanol branching ratio is taken as 50%, so the ethanol quantum yield is 0.015 mol ethanol/mol photons. The E_k value for the results shown is taken as 100 µmol photons/m^2-sec. Calculations assume a 12 hour daylight at the indicated incident irradiance level (E_0, totally absorbed).

Inclusion of biomass productivity, branching ratios, and respiration

We have described above a simple model for calculating the irradiance dependence of the areal rate of fixed carbon production. This is useful for assessing and understanding the potential of a given organism for ethanol or biomass production, the most important element of that assessment being the ethanol productivity. However, the sustainability of that productivity over relevant time periods (months in most scenarios) is a critical factor for commercial application. Biomass productivity is also important, whether we are viewing it as a waste product or a potential product stream. Therefore, measurement and understanding of the time evolution of ethanol and biomass productivities have been important aspects of Algenol's research and development process.

For illustrative purposes, we consider a wild type biomass growth curve, biomass vs time. The accumulation of biomass, under constant average light exposure (could be constant light, or constant light after averaging over the daily photoperiod) will increase super-linearly at early times (exponential growth phase) due to incomplete light absorption at low biomass concentrations. That phase is very short lived or non-existent for most of our cultures, i.e., most of the time our cultures are optically dense after the initial inoculation. The biomass (measured on a daily basis) then rises approximately linearly with time until it begins to reach a plateau, the

so-called “stationary” phase. In that phase, photosynthetic carbon fixation balances the carbon demand for respiration and biomass accumulation ceases. The irradiance at which photosynthesis balances respiration demand is called the compensation light intensity or the compensation point. The plateau can be understood by noting that respiration depends on the total biomass in the culture, whereas photosynthesis (to first order at least) does not, because all the light is absorbed. This is a simple view, and there may be other factors that come into play, but this simple picture has worked very well for our systems thus far. We will now consider the situation for an ethanologenic species, as depicted schematically in Figure Mod-2, and introduce the branching ratio concept. As shown in the figure, the branching ratio (φ), as modulated by the engineered capacity of the EtOH synthesis pathway, is typically in the range 15%-85% for our organisms, most easily determined at short times (before respiration complicates the analysis). Note that φ (referred to as the “fundamental” branching ratio) is based on rates, not cumulative concentrations, and includes the rates for all known dispositions for the fixed carbon (ethanol, cell biomass, non-ethanol dissolved organic carbon, and respiration). It also includes the requirement of 3 fixed carbons per ethanol molecule, because of the loss of one “fermentation” CO_2 molecule in the PDC catalyzed pyruvate to acetaldehyde step. φ is not the same as the experimental carbon branching ratio (CBR) which is based on cumulative concentrations and is impacted by both respiration, acclimation and ethanol quenching effects. CBR also does not usually take into account the fermentation CO_2 loss.

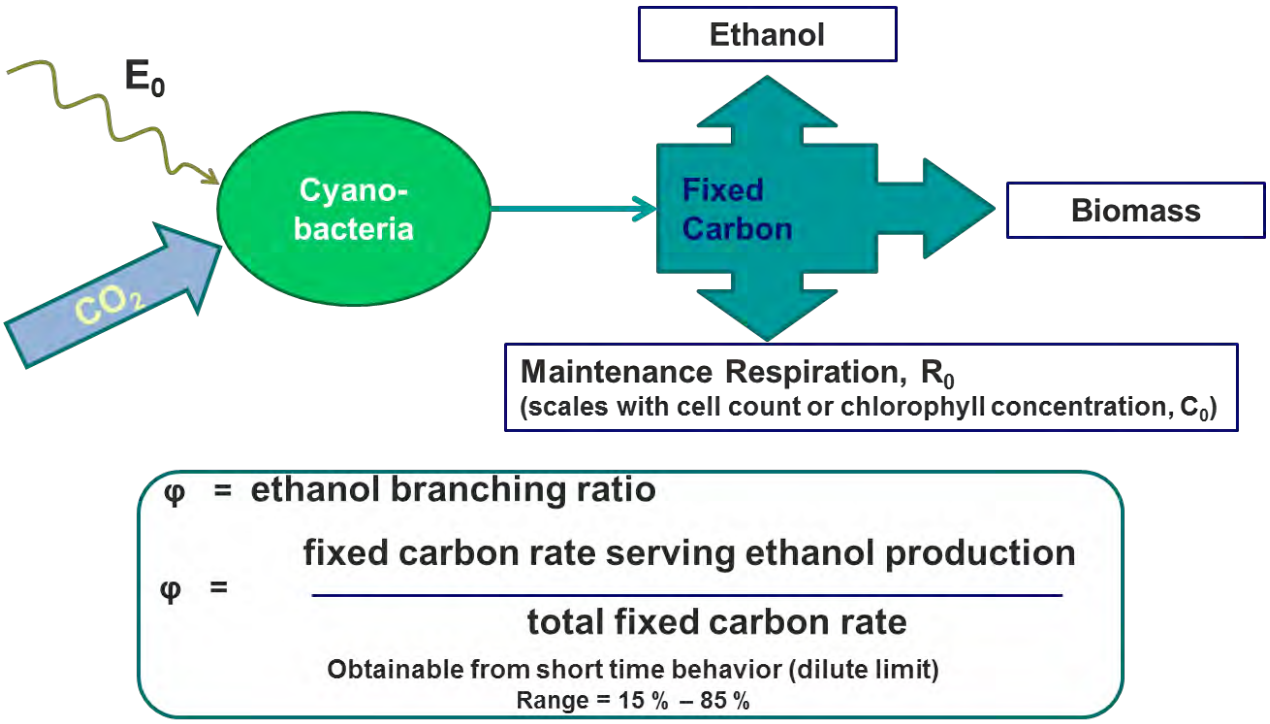


Figure Mod-2. Schematic of the Photobiology Model including respiration. The branching ratio (φ) is a ratio of production rates, not cumulative concentrations.

We have established that the total carbon fixation rate is the same, or nearly the same, for wild type and ethanologenic organisms provided respiratory effects are small (e.g., for early stage cultures) and taking into account the need for 3 fixed carbons in the ethanol pathway. Thus we expect the biomass growth rate to be reduced by a factor of 1- φ for the ethanologenic organism in comparison to the wild type parent. That is consistently observed, the only obvious exceptions being systems undergoing genetic reversion to the wild type. In our analyses, we treat φ as constant that can be calculated from short time behavior of the culture (though see

later discussion of ethanol quenching effects). An upper limit, but close approximation, for φ can be obtained from on-line GC measurements (2 ml scale).

With that background, we can specifically describe both ethanol and biomass production and include respiration effects. The carbon fixation rate for the ethanol pathway (P_e) is

$$P_e = \alpha \varphi E_k \ln(1 + E_0/E_k) \qquad (15)$$

where α is the quantum yield for carbon fixation (mol Cfix/mol photons). The net biomass production includes both dry weight (DW) and non-ethanol dissolved organic carbon (DOC) and is given by

$$P_b = \alpha (1- \varphi) E_k \ln(1 + E_0/E_k) – R_0 C_0 D \qquad (16)$$

where the first term is the total biomass areal production rate (not counting ethanol) and the second term is the respiration term computed on an areal basis. R0 is the specific respiration rate, which is often written as mol O_2 per cell or some other cell count unit since R_0 is usually determined from O_2 consumption in the dark. We will use instead units based on carbon consumption (μmol C_{fix}/mg Chl.a- min). (The difference is the photosynthetic quotient, mol O2 /mol C_{fix}, which is a number in the 1.1 – 1.4 range). C_0 has units of mg Chl.a /volume, the chlorophyll reference being a surrogate for biomass, and for our purposes here is calculated from the DW (ash free dry weight), TOC (total organic carbon), or OD750 based on established relationships and corrections.

Though there are many potential changes in cell metabolism in response to sustained changes in irradiance, the apparent manifestation of those changes, within the context of our phenomenological model, is a change in the photosaturation parameter, E_k, which has irradiance units (μmol photons/m^2-sec). This parameter describes productivity response of the organism (e.g., fixed carbon, O_2 evolution, ethanol) to irradiance (E). Photosaturation occurs whenever the photons are being absorbed by the photosynthetic system faster than they can be processed into product. Thus E_k will have some proportionality to the rate constant for the rate limiting photosynthetic step. In most constructions of the problem (including the Michaelis-Menten formulation) when E = E_k, the productivity is reduced by a factor of 2 with respect to the limiting value at high E (P_{max}). E_k is defined from, and derived for, short-time scale experiments, i.e., for time scales which are thought to be much shorter than those for acclimation. However, there is considerable uncertainty in what that time scale is, and we are reasonably certain from our own experiments, and simple logic, that it depends on the overall biomass production rate. The photoacclimation process results from a response of the organism to the average light level experienced by the organism (which is probably just a convenient surrogate for production rate), and is manifested phenomenologically as a variation in E_k (and P_{max}) In fact, as evidenced by studies of marine ecosystems, E_k will tend to approach, at least approximately, the numerical value of the average light exposure for the cells. Thus long term exposure to high average irradiance per cell (below damage thresholds) will yield a high E_k, and vice- versa for low light. These effects are clearly evident in our cultures (and well known in the literature) and have been shown many times in lab experiments on both WT and ethanologenic organisms. The impact of E_k on productivity curves is included by varying E_k based on its established relationship with the average light, E_0kD (for complete absorption). A time constant of 1-2 days is usually assumed for the photoacclimation, but that choice does not materially affect the results.

For the present purposes, we will define R_0 as a constant, the same night or day, which is equivalent to taking a daily average value that is unchanged during culture aging. It remains to be seen whether or not this is a useful approximation over long time scales. Equations (15) and (16) can be used to model ethanol and biomass measurements as a function of time, extracting estimates of α, φ, and R_0, all of which can be tested for reasonableness based on the literature

and independent experiments. This model has been widely applied to Algenol's data base, at all scales from 2ml PBRs to 100L PBRs. The fitting process and a series of examples is given below.

The final element that enters the analysis procedure is ethanol quenching. Not unexpectedly, as the ethanol concentration increases in the culture. Since ethanol production is affected by the presence of ethanol in the culture, we introduce an ethanol quenching term. We construct this term in a manner similar to fluorescence quenching and take into account the lack of any effect on biomass production (as established in laboratory experiments). The best view of the quenching experiments to date suggests that α is not affected by the presence of ethanol (at the levels relevant herein); thus, reduction in the ethanol production due to ethanol quenching will produce a corresponding increase in biomass production. The form assumed is:

$$\varphi = \varphi_0 \, (1/1+ Q_e \, C_e) \qquad (17)$$

where Q_e is the quenching constant (v/v%$^{-1}$) and C_e is the ethanol concentration in the culture (v/v%). This form meets all the criteria outlined above and has the correct limiting behavior. The constant Q_e is estimated at 0.5 v/v%-1 from recent ethanol spiking experiments. With this value, the ethanol production rate is reduced by a factor of 2 at a concentration of 2 v/v%. At 0.3 v/v%, the expected reduction in φ is about 15%. Thus the effect for the experiments of concern herein is relatively small as ethanol concentrations are usually no larger than 0.4% for the relevant times of our experiments (field batch times are typically ~21 days). Also is should be noted that the correction should be based on the actual concentration in the culture, not the VLE (Vapor-Liquid-Equilibrium) corrected value (See Cultivation discussion). Recent work on organisms with enhanced ADH activity show lowered ethanol quenching effects as would be expected from a kinetic point of view.

The data analysis proceeds as follows:

Step 1 – we calculate the total net fixed carbon (net C_{fix} on an areal basis). This will be the sum of the dry weight carbon plus ethanol carbon x 1.5 (C_{et}) plus other dissolved organic carbon (DOC), or TOC plus half the carbon in ethanol so as to include the "fermentation" CO_2. C_{et} is the fixed carbon in ethanol, as measured directly and corrected for losses to the headspace due to vapor liquid equilibrium (VLE) considerations. (All quoted C_{et} values in this section are VLE corrected; the corrections are generally small.) The 1.5 multiplier takes into account the fermentation carbon. We rely on correlations of OD750 to TOC – ethanol for the dry weight and DOC. The net C_{fix} calculated is then: net C_{fix} = TOC + C_{et} /3. The OD750 measurement generally provide a good surrogate for the non-ethanol portion of TOC. This procedure provides a net C_{fix} versus time curve. We fit this curve to the cumulative net C_{fix} calculated from the sum of Equation (16) with φ set to zero, which is equivalent to adding Equations (15) and (16) to obtain the total net fixed carbon rate. E_k is calculated based on the evolution of the average light E_o/kD during the experiment (k being measured daily, and E_o continuous monitored outdoors/controlled indoors). The respiration rate (R_0C_0D) functions with the same R_0 for the entire experiment; this is equivalent to assuming the same maintenance (or basal) respiratory rate during night and day. The model fit from these steps provides the first estimate of α and R_0. The fitting is evaluated on visual basis in all cases, which is judged adequate for our current purposes and precision requirements for the derived parameters.

Step 2 – a graph of C_{et}, the fixed carbon required for the ethanol pathway, versus culture time is constructed. Obviously, this step is omitted for WT organisms. That curve is fit with Equation (15) using the α value from the first step. The only variable here is φ. This is a consequence of the model construction where we assume respiration does not compete with ethanol production. Generally, we recycle into Step 1 testing alternative values for the three parameters until we are satisfied that we have achieved a good fit.

Step 3 – we compare to the biomass alone, which is generally addressed in the fitting process as the surrogate OD750nm. If the fit is judged inadequate or poor, we will recycle to the extent necessary.

An example for an outdoor PBR experiment is shown in Figure Mod-3. This experiment was conducted in Fort Myers, Florida with a strain (ABCC:1535) in use in 2012, which had an ethanol branching ratio of only about 40%. The experiment was conducted in the fall of 2012. Thus, the irradiance (E_0) was generally declining over the 70 day period of the experiment.

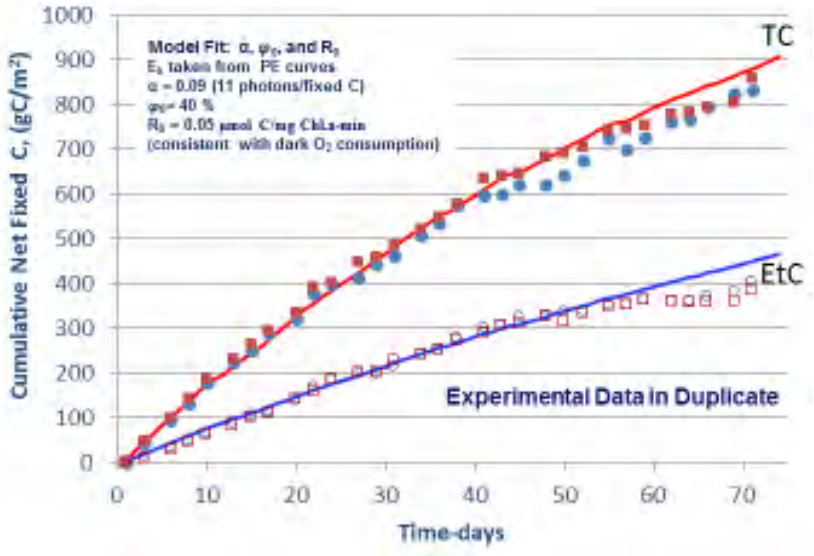


Figure Mod-3. Typical modeling fitting results for total fixed C (strain 1535, outdoor cultivation, circa 2012). Note that the branching ratio for this organism is only about 40%. Note that the experiment extended to 70 days with no hint of model breakdown before about 50 days.

- Fit parameters: α = 0.090 C_{fix}/photon, ϕ_0 =75%, R_0= 0.08 µmol C/mg Chl.a-min
- *Same parameter fit for all 3 irradiances*

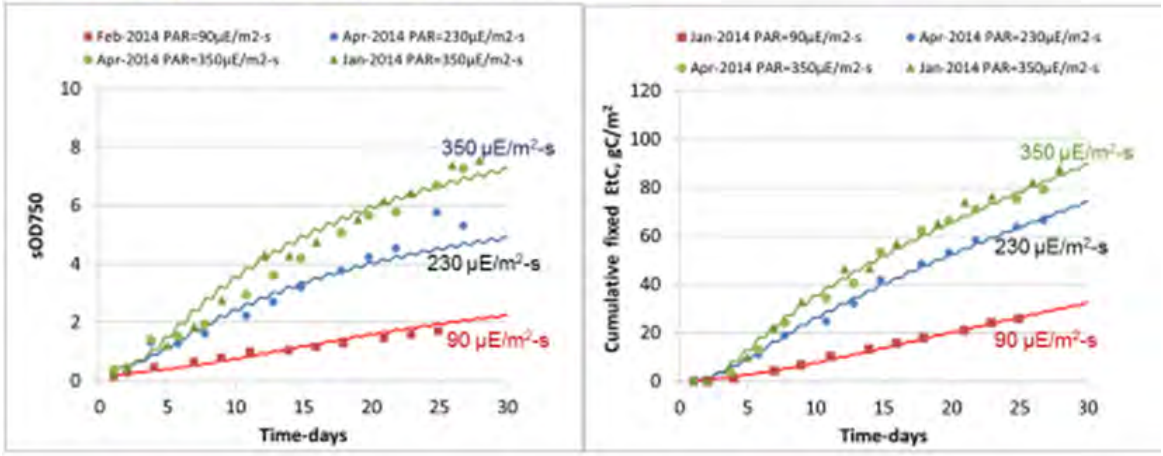


Figure Mod-4. Productivity Modeling of indoor experiment dataset: biomass and ethanol productivity.

Indoor experiment productivity modeling

In Figure Mod-4, the productivity model fitting analysis is applied for a series indoor experiments for the strain deployed at the IBR. Data (average of 3 PBRs) show biomass (OD750 surrogate) and ethanol accumulation in indoor PBR at different irradiance levels: 90,

230 and 350 μE/m^2-s. The latter two values correspond annual irradiance levels for height-to-spacing ratios of 4:1 and 2.4:1, respectively. The model parameters are: α = 0.09 molC/mol photon, φ_0=75%, R_0 =0.08 μmolC/mg Chl.a-min, where φ_0 is the initial fundamental ethanol branching ratio at very low ethanol concentration. With these parameters and including fixed parameters shown in the figure, the productivity model provides an excellent representation of both biomass and ethanol accumulation. As ethanol concentration increase, the ethanol branching ratio apparently decreases with that decline adequately described by the quenching term as $\varphi/\varphi_0 = 1/(1+ Q_eC_e)$. Q_e=0.5 was used for strain 1658 for all model fitting analysis. Figure Mod-4 shows the productivity model can represent indoor experiment data quite well with a single set of photosynthetic parameters covering a fairly large range in irradiance conditions that are relevant to outdoor conditions in Florida.

Outdoor experiment productivity modeling

In Figure Mod-5, we applied this productivity model for over 50-d outdoor algae cultivation (May-June 2013) with an organism that is very similar in performance to the organism used for the IBR deployment. The data were fit as described above using C_{fix} (see Figure Mod-6) but it is interesting to inspect the actual model fits versus the actual measurements (Figure Mod-5a). With daily insolation (PAR), and similar model parameters as indoor experiments: α = 0.09 molC/mol photon, φ_0=72%, R_0 =0.05 μmolC/mg Chl.a-min, we are able to describe experiment data very well for biomass and ethanol production over 30-d period. After 30-d, we can still project ethanol production very well but the biomass fit starts to deviate. There are many potential reasons for this, but the deviations are not that large (given the 72% branching ratio). Also, we are mainly interested in fastest ethanol production period, i.e., the first 20 or so days.

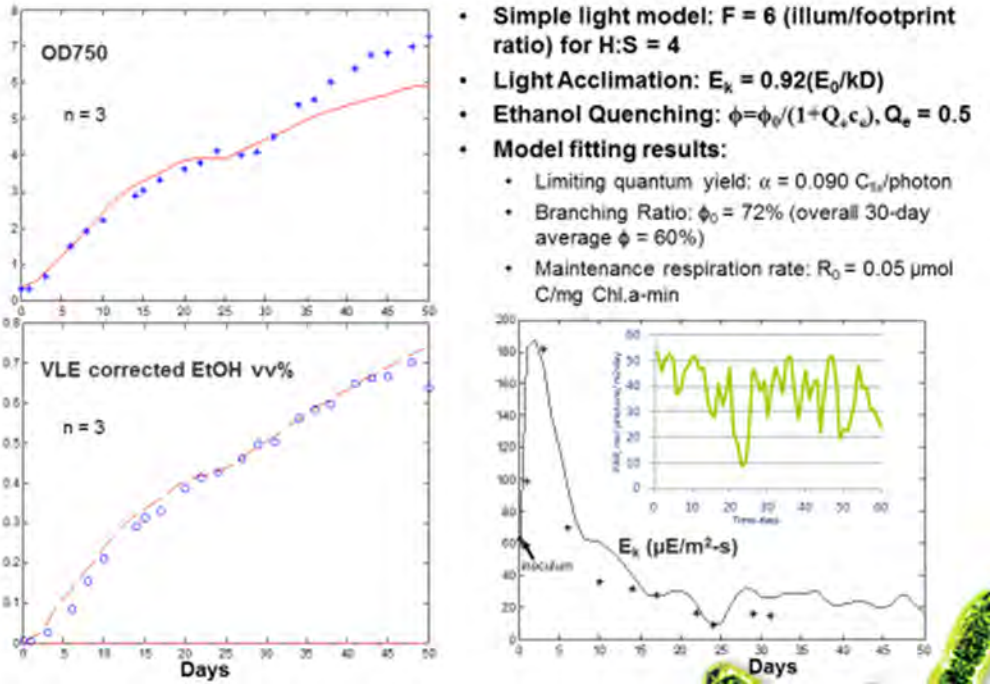


Figure Mod-5. Productivity modeling of outdoor experiment dataset: biomass and ethanol productivity (strain 1578 in 2003).

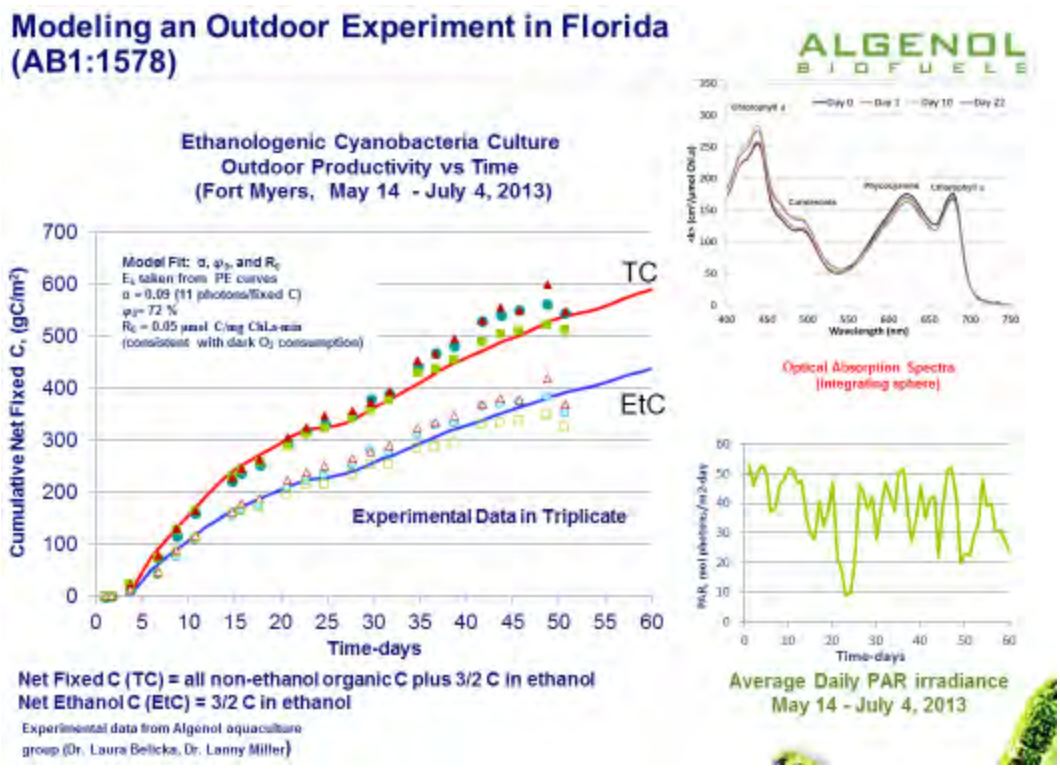


Figure Mod-6. Productivity modeling of outdoor experiment dataset: biomass and ethanol productivity.

In one of the insets we show the E_k results from PE experiments conducted on samples drawn from the Figure Mod-5 experiment on the days indicated. For consistency, we use the same physical description of the system for the PE curve analysis, i.e., a Michaelis-Menton formulation similar to Equation (2), but including respiratory effects. The initial E_k value at zero time is the result for the inoculum. That value is low because of the low average light (dense culture) employed in the inoculum preparation. When diluted in the PBR, the organism reacts to the higher light levels by rapidly increasing its E_k value. P_{max} follows the same pattern as the limiting quantum yield, α, is more or less unchanged (~0.08-0.09) throughout the experiment. The E_k value peaks out at about 200 μE/m^2-s and then declines to very low values over the next several weeks (down to ~20 μE/m^2-s). This behavior is seen consistently in both indoor and outdoor experiments. The largest E_k value we have observed in outdoor experiments is about 350 μE/m^2-s, which is very close to the annual average light level for Florida (averaged also over the PBR surface). These results are plotted in Figure Mod-7 versus the average light E_0/kD. Included in Figure Mod-7 are the results of companion experiments conducted at the same time and same manner as the ethanologenic experiments, except with WT AB1. This large difference between WT and ethanologenic is not well understood, but is consistently seen in indoors and outdoors. It may well be more appropriate to scale versus metabolic rates for biomass production which would essentially eliminate the difference between the curves since WT is about 3x the ethanologenic organism in terms of biomass production.

In any case, this decrease in E_k with culture density leads to a corresponding decrease in productivity and, within the context of this model, is the main loss factor for ethanol production.

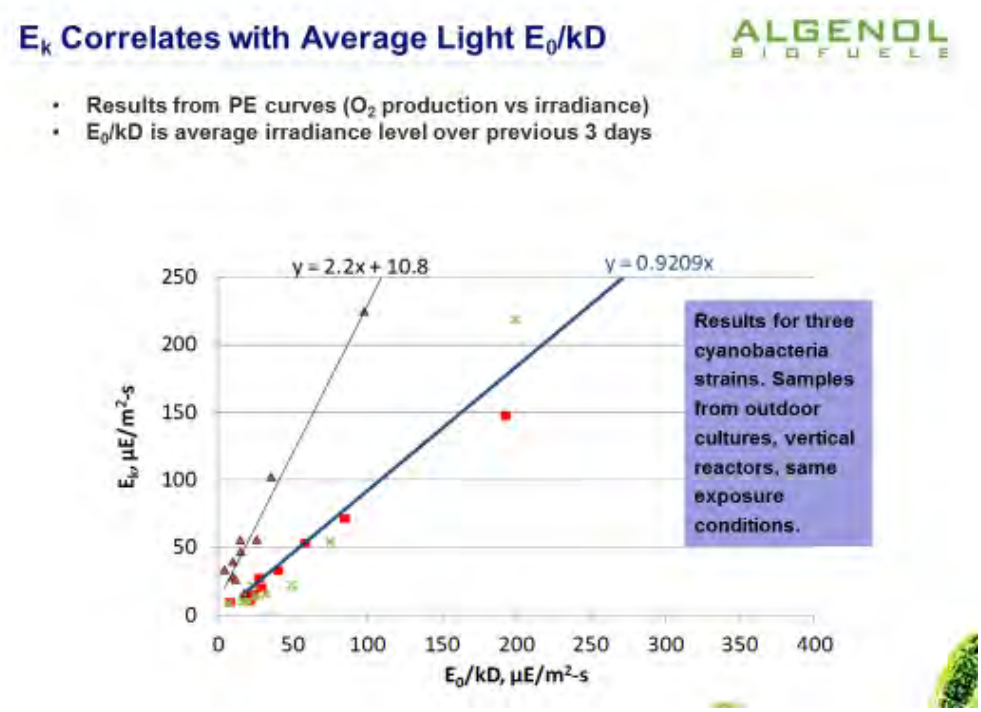


Figure Mod-7. E_k correlation with average light E_0/kD; triangles are wild type and other symbols represent ethanol producing strains.

Productivity annualization: indoor to outdoor

The productivity model has been used for a series indoor and outdoor experiment data analysis, and using the photosynthetic parameters and Annual Florida PAR data, we are able to predict annualized productivity.

The annualization process steps are:

1. Inputs PBR array configuration, solar configuration, solar irradiance, and photosynthetic parameters
2. Irradiance (E_0) from Solar Anywhere Database (typically use the average of last 20 years)
3. Photosynthetic parameters from lab and outdoor experiments on strains under consideration
4. Outdoor performance data (Productivity, batch length, and calendar date for the experiments) analyzed for any anomalies (e.g. contamination events, extreme weather, etc.

The types of curves that result from this process are shown in Figure Mod-8. 14 day rates are higher than 30 rates because of the photosaturation effects discussed above, mainly. These data are for a moderate height to spacing ratio. Results for wider spacing chosen for commercial design are about 12% lower, but adding in the biofuel production from the biomass residue adds about 15% (to get to TGOLF). Thus, we can expect from this total data set an annualized productivity of between roughly 5600 and 6800 TGOLF, likely closer to 5600 since longer batch cycles are favored for ethanol production.

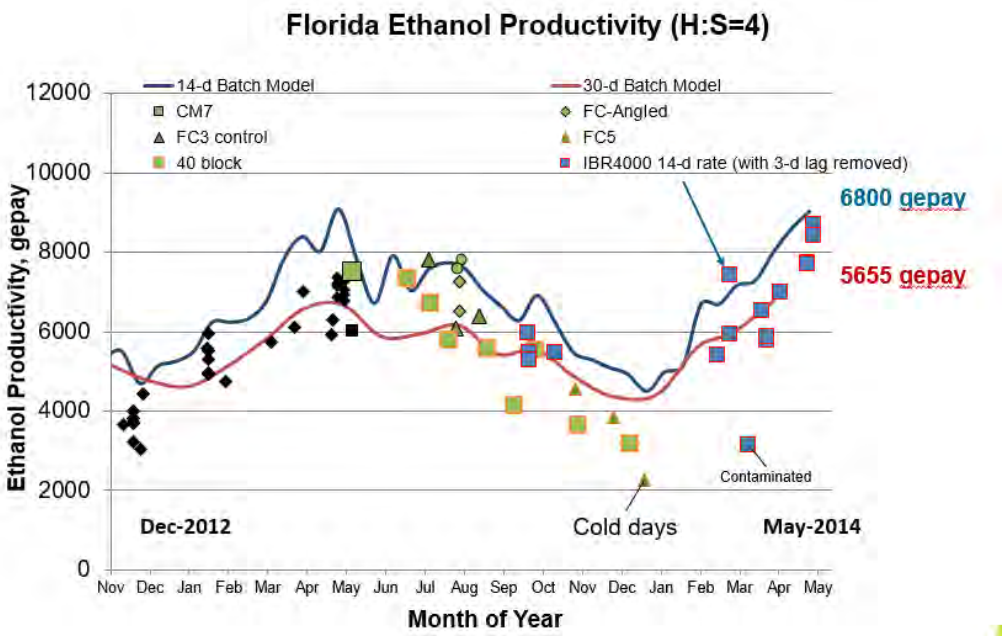


Figure Mod-8. Florida Ethanol Productivity Annualization (H:S=4). Over 100 data sets area represented in this curve.

Table Mod-1. Summary of productivity modeling for annual productivity prediction (H:S=2.5).

Relation between Experimentally Derived Photosynthetic Parameters and Annual Productivity (H:S = 2.5)

ALGENOL BIOFUELS

- Simple Model (F=4 for H:S=2.5,10ftvPBR at FL annual average light)
- Fixed Parameters: E_c = 0.92 (E_0/kD) from CM7 (AB0004, 1578), Q_c =0.5

Strain	Exp.	α	ϕ_0	R_0	14-d Gepay/Tgolf	30-d Gepay/Tgolf
AB0015 (TK293)	outdoor (CM7)	0.090	58%	0.050	4590/5700	3760/4590
AB0004 (1578)	outdoor (CM7)	0.090	72%	0.050	5930/6850	4850/5575
AB0007 (1658)	indoor	0.090	75%	0.080	6300/7125	5230/5840
AB0007 (1658)	outdoor (FC6.1)	0.075	70%	0.100	5140/5860	4390/4900
AB0007 (1658)*	outdoor (FC7.3)	0.065	72%	0.100	4780/5390	4150/4600
AB0010 (1933)	FL indoor	0.078	84%	0.050	6450/7090	5390/5950
AB0010 (1933)*	outdoor (FC7.3)	0.060	78%	0.080	4890/5415	4280/4700
AB0012 (1938)	FL indoor (Urea)	0.092	75%	0.100	6450/7245	5380/5960
AB0012 (1938)	FL indoor (Nitrate)	0.092	78%	0.100	6750/7510	5640/6200
AB0012 (1938)*	outdoor (Nitrate)	0.060	80%	0.080	5020/5525	4400/4820

*Values distorted due in part to Tropical Storm Bertha

In Table Mod-1, we summarize photosynthetic parameters from a set of indoor and outdoor experiments that we view as reasonably well-behaved, e.g. no obvious contamination problems or other outlier problems. The predicted ethanol productivity (GEPAY) and total biofuel productivity (TGOLF) are shown. With comparison of indoor and outdoor annualized rate, indoor

total fuel production rate range from 5800 (30-d) to 7000 TGOLF (14-d) for strain 1658 (the IBR strain), while outdoor rate is 4800 (30-d) to 5600 TGOLF (14-d) (average values from FC6.1 and FC7.3). The total biofuel production rate is directly related to batch length. To achieve economically viable levels of culture EtOH, the Algenol IBR operation is currently a 21-d batch, therefore the expected rate will be between 4800 to 5600 TGOLF, with somewhat higher results expected from earlier experiments at reasonable scale, but outside the IBR setting.

Scenarios for improved performance (setting P90, P50, and P10)

Based on the above, as well as the compilation of results earlier in this section, we suggest a P90 value of 5600 TGOLF is a reasonable estimation for how the current system would perform under normal operating conditions. P90 is discussed in more detail in the TEA section, but is roughly the current status of a particular parameter or more specifically the performance one can expect to achieve at 90% confidence on the time scale of commercialization. That value will form the basis for the TEA analysis productivity input and the basis for suggesting how improved biological performance and engineering innovations could lead to improved performance. P50 and P10 have similar probabilistic definitions, and obviously increased uncertainty.

Some approaches to improved performance:

1. If photosaturation effects could be eliminated, i.e., if we could lock in an E_k of 300 μE/m^2-s, the 5,600 TGOLF value would become approximately 9,000 or a little greater. Even locking in an E_k of 150 μE/m^2-s, yields about 8000 TGOLF.
2. If the ethanologenic organism behaved the same way to average light as the wild type organism (i.e., use the WT curve in Figure Mod-7 for the prediction), the 5,600 TGOLF would become about 7,000 TGOLF.
3. If ethanol quenching could be eliminated, the 5600 TGOLF value would become about 7000 TGOLF. It should be noted that laboratory experiments have been done that show a great reduction in ethanol quenching with higher ADH activity, but these organisms have not been scaled up for outdoor deployment.
4. Finally from an engineering perspective, semi-continuous operation (analogous to that already demonstrated for biomass-only production in our labs) could yield about 7,000 TGOLF. Similar improvements could be achieved if the light could be more uniformly distributed over the PBR surface.

In summary, there are a number of avenues for improved productivity. We have focused thus far on 1) and 2) and have not been as successful as anticipated in delivering significant improvements in ethanol production, although some success has been achieved in increasing biomass. It is likely that 3) will deliver some improvement but not likely that full elimination of quenching is possible. There is some hope for item 4) and that will likely be pursued as a spinoff of our current program aimed at improved biocrude production. Biological approaches to 1) and 2) have not been exhausted and could yield improvements significant enough to warrant taking the next steps towards system development.

Regarding P90, P50, and P10 values for the TEA analysis, we have chosen 5,600, 7,300, and 9,100 TGOLF based on the above reasoning and the input from our subject matter experts. We regard these as reasonable estimates that are certainly possible from what we know today about the systems of interest here.

Publications, Presentations and Awards

Peer Review Publications

1. D. Luo, Z. Hu, D. Choi, V.M. Thomas, M.J. Realff, and R.R. Chance, "Lifecycle Energy and Greenhouse Gas Emissions from Ethanol Produced by Algae," *Environmental Science and Technology* **44**, 8670-8677 (2010).
2. R.P. Lively, M.E. Dose, J.A. Thompson, B.A. McCool, R.R. Chance, W.J.Koros, "Ethanol and water adsorption in methanol-derived ZIF-71," *Chem. Comm.* **47**, 8667-8669 (2011).
3. J. D. Noel, W.J. Koros, B.A. McCool, and R.R. Chance, "Membrane-Mediated Delivery of Carbon Dioxide by Photoautotrophs: Eliminating Thermal Regeneration in Carbon Capture," *Ind. Eng. Chem. Res*. **51** 4673-4681 (2012).
4. K. Zhang, R.P. Lively, J.D. Noel, M.E. Dose, B.A. McCool, R.R. Chance, and W.J. Koros, "Adsorption of Water and Ethanol in MFI-Type Zeolites," *Langmuir* **28**, 8664-8673 (2012).
5. K. Zhang, R.P. Lively, M.E. Dose, L. Li, W.J. Koros, D.M. Ruthven, B.A. McCool, and R.R. Chance, "Diffusion of Water and Ethanol in Silicalite Crystals Synthesized in Fluoride Media," *Microporous and Mesoporous Materials* **170**, 259-265 (2013).
6. K. Zhang, R.P. Lively, M.E. Dose, A.J. Brown, C. Zhang, J. Chung, S. Nair, W.J. Koros, and R.R. Chance, "Alcohol and Water Absorption in Zeolitic Imidazolate Frameworks," *Chemical Communications* **49**, 3245-3247 (2013).
7. K. Zhang, R.P. Lively, C. Zhang, W.J. Koros, and R.R. Chance, "Investigating the Intrinsic Ethanol/Water Separation Capability of ZIF-8: an Absorption and Diffusion Study," *Journal of Physical Chemistry C* **117**, 7214-7225 (2013).
8. R. P. Lively, P. Sharma, D. Luo, B. McCool, J. Beaudry-Losique, V. Thomas, M. Realff, and R. R. Chance, "Anthropogenic CO_2 as a feedstock for the production of algal-based biofuels," *Biofuels, Bioproducts, and Biorefining* **9**, 72-81 (2015).
9. K. Eum, K. Jayachandrababu, F. Rashidi, K. Zhang, J. Leisen, S. Graham, R. Lively, R. Chance, D. Sholl, C. Jones, and S. Nair, "Highly tunable molecular sieving and adsorption properties of mixed-linker zeolitic imidaxolate frameworks," J. Amer. Chem. Soc. **137**, 4191-4197 (2015).

Invited Presentations

1. R.R. Chance, "Biofuels: Ethanol from Algae," Department of Materials and Environmental Chemistry, Stockholm University, Stockholm, Sweden (October 8, 2010)
2. R.R. Chance, "Direct to Ethanol: Production of Ethanol from Algae," Edison College, Fort Myers, FL (October 29, 2010)
3. B.A. McCool, "Algenol – Engineering Group," Florida Gulf Coast University, Fort Myers, FL (November 18, 2010)
4. B.A. McCool "Algenol – Technology Overview and Engineering Group Initiatives," NREL, Golden, CO (December 1, 2010)
5. B.A. McCool, "Algenol – Direct to Ethanol Process and Life Cycle Analysis," Sustainability Lecture, Florida Gulf Coast University, Fort Myers, FL (February 21, 2011)
6. R.R. Chance, "A Cyanobacteria-Based Photosynthetic Process for the Production of Ethanol," National Research Council, Washington, DC (June 13, 2011)

7. W.P. Porubsky, “A Cyanobacteria-Based Photosynthetic Process for the Production of Ethanol,” Center for Bioenergy and Photosynthesis, ASU, Tempe, AZ (February 16, 2012)
8. H.L. Miller III, “Sun to Ethanol: biofuel production from cyanobacteria photosynthesis,” Marine Science Department, University of Georgia (March 12, 2012).
9. R.R. Chance, “Advanced Biofuels - Creating a New Business,” Delta State University, Cleveland, MS (April 20, 2012)
10. R.R. Chance, “A Cyanobacteria-Based Photosynthetic Process for the Production of Ethanol,” Wageningen University, The Netherlands (December 11, 2012)
11. R.R. Chance, “A Cyanobacteria-Based Photosynthetic Process for the Production of Ethanol,” Georgia Tech, Atlanta, GA (February 20, 2013)
12. P. Sharma, “Algenol – Direct to Ethanol Technology Overview and Life Cycle Analysis,” SCHB Division at ACS Annual Meeting, New Orleans, LA (April 8, 2013)
13. R.R. Chance, “Anthropogenic CO_2 as a Feedstock for Cyanobacteria-Based Biofuels,” International Conference on CO_2 Utilization, Alexandria, VA (July 24, 2013)
14. W. P. Porubsky, “A Cyanobacteria-Based Photosynthetic Approach for the Production of Ethanol Algenol’s Direct to Ethanol® Process,” Society for Industrial Microbiology 2013 Annual Meeting, San Diego, CA (August 15, 2013)
15. R.R. Chance, “Engineering and Techno-Economic Modeling: CO_2 Utilization and Global Warming,” Florida Gulf Coast University, Fort Myers Florida (September 25, 2013)
16. H.L. Miller III. “Algenol’s Second Generation Photobioreactor,” ABO Algae Biomass Summit, Orlando, FL (October 3, 2013).
17. R.R. Chance, “Realizing the Full Potential of Algal Biofuels,” Rutgers University, New Brunswick, NJ (December 2, 2013)
18. R.R. Chance, “Anthropogenic CO_2 as a Feedstock for Cyanobacteria-Based Biofuels,” Dreyfus Lecture, Chemistry Department, Dartmouth College (May 13, 2014)
19. R.R. Chance, “Integration of Biofuel Production with Power Generation Facilities,” Algal Biomass, Biofuels, and Bioproducts Meeting, Santa Fe, New Mexico (June 17, 2014)
20. P. Roessler, “Photosynthetic production of biofuels from CO_2 by cyanobacteria using Algenol’s Direct to Ethanol® process,” Society for Industrial Microbiology and Biotechnology (SIMB) Annual Meeting, St. Louis, MO (July 22, 2014)
21. P. Roessler, “Photosynthetic production of biofuels from CO_2 by cyanobacteria using Algenol’s Direct to Ethanol® process,” 4th International CeBiTec Research Conference, Bielefeld, Germany (September 21, 2014)
22. P. Roessler, “Photosynthetic production of biofuels from CO_2 by cyanobacteria using Algenol’s Direct to Ethanol® process – Strain development aspects,” ABO Algae Biomass Summit, San Diego, CA (October 1, 2014)
23. Y. Yuan, “A Cyanobacteria-Based Photosynthetic Process for Bioethanol Production: Modeling of Productivities in Laboratory and Outdoor Photobioreactors,” ABO Algae Biomass Summit, San Diego, CA (October 1, 2014)
24. R.R. Chance, “A Cyanobacteria-Based Photosynthetic Process for Bioethanol Production: Modeling of Productivities in Laboratory and Outdoor Photobioreactors,” University of New Mexico, REAP Meeting, Las Cruces (February 20, 2015)

25. R.R. Chance, "Anthropogenic CO_2 as a Feedstock for Cyanobacteria-Based Biofuels," American Physical Society Meeting, Baltimore, MD (April 12, 2015)
26. R.R. Chance, "Anthropogenic CO_2 as a Feedstock for Cyanobacteria-Based Biofuels," University of Georgia, Athens, GA (May 5, 2015)
27. R.R. Chance, "Anthropogenic CO_2 as a Feedstock for Cyanobacteria-Based Biofuels," Bioenergy 22015, Washington, DC (June 24, 2015)
28. R.R. Chance, "The Algenol Biofuel Process: Sustainable Production of Ethanol and Green Crude," Green Chemistry Meeting (EPA and ACS sponsored), Washington, DC (July 14, 2015)
29. H.L. Miller III. "Biological and Photobioreactor Evolution with Implications to Direct-to-Ethanol® Technology," DOE Algal Biology Toolbox Workshop, San Diego, CA (May 24, 2016)
30. R.R. Chance, "Carbon Capture and Utilization in Algae-Based Biofuel Production," Van't Hoff Lecture, Delft University, The Netherlands (June 20, 2016)
31. R.R. Chance, "Carbon Capture and Utilization in Algae-Based Biofuel Production," DOE National Energy Technology Laboratory, Pittsburg, PA (July 28, 2016)
32. P. Roessler, "Algenol's Direct-to-Ethanol process: CO_2 + sunlight + cyanobacteria = ethanol," Gordon Research Conference on Green Chemistry, Stowe, VT (August 2, 2016)

Awards

1. 2013 Florida Governor's Innovators in Business Award
2. PLATTS 2014 Global Industry Leadership Award for Biofuels
3. PLATTS 2015 Breakthrough Solution of the Year
4. 2015 Presidential Green Chemistry Challenge Award
5. Participant in 2015 US Presidential Trade Mission to China
6. US Department of Energy 2016 Algal Biomass Grant ($5 million)